IMMUNOTHERAPY OF METASTATIC DISEASE

Dissertation

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Summary

Despite recent advances in oncology, metastatic disease remains the major cause of cancer-related death. The cascade of events involved in this process includes invasion of surrounding tissue, intravasation into the blood or lymph vasculature, survival in the circulation and extravasation and colonization of distant tissue. Each of these steps involves crosstalk between tumour cells and hematopoietic cells, including lymphocytes and different cell types of myeloid origin. Whereas most T cell subsets and NK cells contribute to tumour control, tumour-associated macrophages in most of the cases promote progression and metastasis.

Using a model of spontaneous metastasis from resected, syngeneic tumours, I have investigated the impact of several cell types and interventions on formation and progression of metastasis.

The first chapter describes that depleting tumour-associated macrophages with an inhibitor of colony stimulating factor 1 receptor (CSF1R) unexpectedly promotes metastasis. CSF1R blockade indirectly diminishes the number of circulating NK cells due to a paucity of myeloid cells that trans-present the survival factor IL-15 to NK cells. CSF1R blockade also abolishes the anti-metastatic effect of agonistic anti-CD40 therapy.

The second chapter describes that combination of agonistic anti-CD40 antibodies with immune checkpoint blockade (anti-CTLA-4 plus anti-PD-1) did not further improve the anti-metastatic effect of anti-CD40. Monotherapy with agonistic anti-CD27 antibodies, which bypasses the CD70-signal provided by activated dendritic cells, did not impact on the development or progression of spontaneous metastasis.

The third chapter shows that the tumour-derived, proinflammatory factor prostaglandin E_2 promotes metastasis. Our results suggest that prostaglandin E_2 may be an interesting therapeutic target.

Finally, I show in the fourth chapter that the adhesion molecule ALCAM contributes to development of metastasis as *Alcam*$^{-/-}$ mice develop less metastases than *Alcam*$^{+/+}$ mice.

The results presented in this dissertation provide further evidence for immune-mediated control of the metastatic disease and may contribute to the development of new anti-cancer therapies.

Zusammenfassung

Trotz der jüngsten Fortschritte im Bereich der Onkologie stellen Metastasen weiterhin die Haupttodesursache für Krebskranke dar. In diesem Prozess kommt es zur folgenden Kette von Ereignissen: die Invasion der Krebszellen in umliegendes Gewebe, das Eindringen in Blut- oder Lymphgefässe, ihr Überleben im Kreislauf, Extravasation, und Kolonisation in entfernten Organen. Jeder dieser Schritte umfasst die Kommunikation zwischen Krebs- und hämatopoetischen Zellen, einschließlich der Lymphozyten und verschiedener Zellen myeloischen Ursprungs. Während die Mehrheit der Untergruppen von T-Zellen und NK-Zellen zur Tumorkontrolle beitragen, unterstützen die Tumor-assoziierten Makrophagen die Krebsentwicklung und fördern Metastasen.

Unter Verwendung eines nach Resektion von syngenen Tumoren entstehenden spontanen Metastasenmodels untersuchte ich die Wirkung mehrerer Zelltypen und Interventionen auf die Bildung und Progression der Metastasen.

Im ersten Kapitel wird gezeigt, dass eine Verringerung der Tumor-assoziierten Makrophagen mithilfe eines Inhibitors des kolonienstimulierenden Faktor 1 Rezeptors (CSF1R) die Metastasenbildung unerwartet unterstützt. Das Blockieren von CSF1R vermindert indirekt die Anzahl der zirkulierenden NK-Zellen aufgrund des Mangels an myeloiden Zellen, die NK-Zellen den Überlebensfaktor IL-15 trans-präsentieren. Das Blockieren von CSF1R hebt ausserdem die anti-metastatische Wirkung der agonistischen anti-CD40 Therapie auf.

Das zweite Kapitel thematisiert, dass die Kombination agonistischer anti-CD40 Antikörper mit Immun-Checkpoint Blockade (anti-CTLA-4 plus anti-PD-1 Antikörper) die anti-metastatische Wirkung nicht verbessert. Die Monotherapie mit agonistischen Antikörpern anti-CD27, welche das von aktivierten dendritischen Zellen zugelieferte Signal CD70 umgeht, beeinflusste die Entwicklung oder das Fortschreiten der spontanen Metastasen nicht.

Das dritte Kapitel zeigt, dass der tumorassoziierte proentzündliche Faktor Prostaglandin E2 Metastasen fördert. Unsere Ergebnisse weisen darauf hin, dass Prostaglandin E2 ein interessantes therapeutisches Ziel sein könnte.

Abschliessend zeige ich im vierten Kapitel, dass das Adhäsionsmolekül ALCAM zur Entwicklung der Metastasen beiträgt, da *Alcam*$^{-/-}$ Mäuse weniger Metastasen bilden als *Alcam*$^{+/+}$ Mäuse.

Die in dieser Dissertation präsentierten Ergebnisse liefern weitere Hinweise für die immunvermittelte Kontrolle von Metastasen und können zur Entwicklung neuer Krebstherapien beitragen.

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Table of Contents

1. Introduction

1.1 Current view on cancer

Advances in health care vastly increased life expectancy, leaving cancer as one of the main threats. In 2012 approximately 14 million new cases have been diagnosed worldwide and this statistic is expected to increase over the next decades (*1*). The number of cancer-related mortalities reached 8.2 million, the majority of which were due to metastatic disease (*1-3*). The most common cancer types in women are breast, colorectal, lung, cervix and stomach, whereas in men lung, prostate, colorectal, stomach and liver (*1, 4*). All these cancers are prone to form secondary lesions, e.g. bone and lung metastases, some of which can develop without symptoms for prolonged time (*5, 6*).

Cancer covers a vast spectrum of diseases that lead to dysplastic changes that can arise in almost every organ. More than 100 distinct types of this disease have been recognized (*7*), but all share the common trait of uncontrolled cell proliferation. This is caused by accumulation of mutations in the genomic and mitochondrial DNA (*8-10*), leading to deregulation of cell death signalling pathways, cell metabolism and elevated expression of normal and mutated, constantly active growth factor receptors (*7, 11-14*). Although uncontrolled proliferation characterizes as well benign tumours, malignant cancers in addition can infiltrate adjacent tissue (*15*).

Solid tumours are composed of cancer cells and stroma, which consists of recruited mesenchymal and immune cells of host origin, collectively known as the tumour microenvironment. The tumour microenvironment often changes with disease progression (*16*) and contains adipocytes, epithelial cells, fibroblasts, pericytes and endothelial cells as well as various immune cells. Each of these cells types can be further divided into subsets playing differential role in cancer development - both restraining the growth of transformed cells, as well as supporting tumours by maintenance of nutrient and oxygen supply, production of growth factors, removal of the toxic metabolites and protection from immunosurveillance (*10, 17*).

1.2 The immune system

The immune system evolved to protect the host against pathogens. Consequently, pathogen-associated molecular patterns (PAMPs) and inflammation are essential cues for activation of the immune system.

The immune system can be divided into two complementary branches – the innate and

adaptive part – both of which are involved and collaborate in the defence against pathogens.

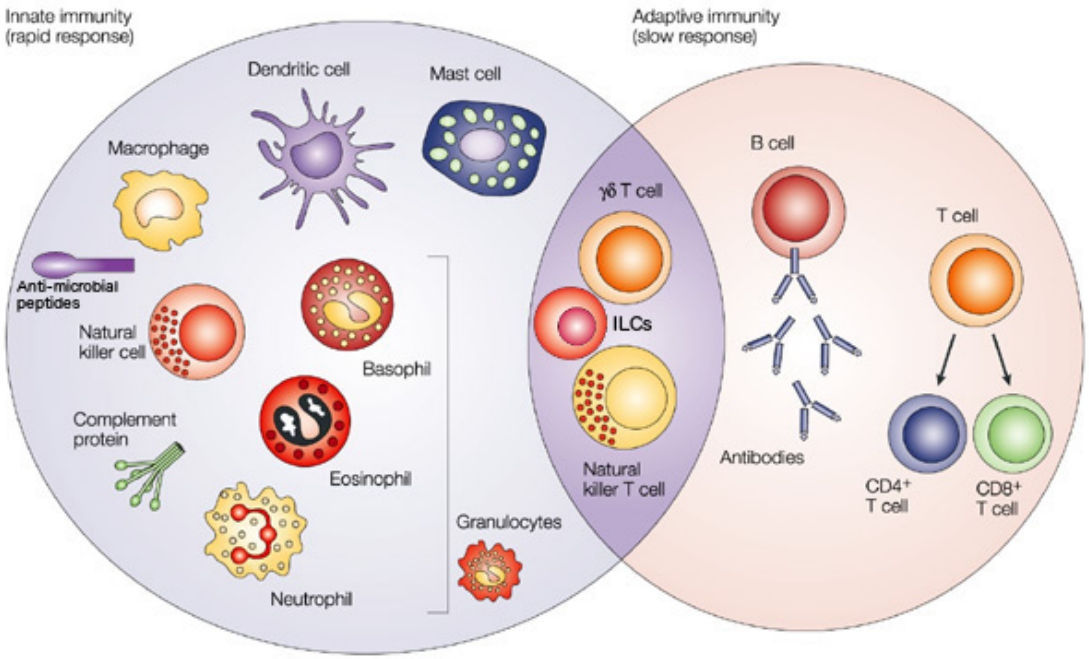

Figure 1. Components of the innate and adaptive immune system. The innate immune system consists of multiple leukocyte populations as well as complement and anti-microbial proteins and peptides. Adaptive immunity consists of T cells and B cells, the latter produce antibodies. NKT cells, γδ T cells and innate lymphoid cells (ILCs) are on the crossroad between the adaptive and innate parts of the immune system. (Figure adapted from: *18*)

1.2.1 Innate immunity

The innate branch of immune system is evolutionary more ancient than the adaptive part and recognises patterns rather that single epitopes (*19, 20*). The activation of innate immunity forms an immediate defence against pathogens and in addition, paves the way for adaptive response (21-23). Epithelial cells but also innate leukocytes such as granulocytes (neutrophils, basophils, eosinophils), mast cells, natural killer (NK) cells, macrophages and dendritic cells (DCs) mediate innate responses and there is an inherent overlap and, to some degree, redundancy between the reactions mediated by these cells.

The main functions of innate immunity include:

- Forming a barrier for pathogens to physically block them from penetrating the tissue;
- Activation of the complement cascade, leading to opsonization and lysis of the targets;
- Killing pathogens though secretion of cytotoxic peptides and proteins;
- Removal of pathogens by phagocytosis;

- Killing own infected cells by induction of apoptosis;
- Attraction of leukocytes of both innate and adaptive immunity;
- Activation of the adaptive immune system by antigen presentation and cytokine production;
- Control of the inflammation by secretion of immune-dampening mediators.

Innate lymphoid cells (ILCs), NK T cells and γδ T cells are on the crossroad between innate and adaptive immunity. ILCs belong to the lymphoid lineage of leukocytes, but they lack T and B cell receptors (TCR and BCR) and can act in a similar way to both T helper cells and NK cells (*24-26*). The TCR of γδ T cells is generally not specific for antigen-MHC complexes, but instead enables recognition of PAMPs (*27*). In a similar manner, the TCR of NK T cells is restricted to a set of lipid antigens, albeit they share many other properties with NK cells (*28*).

1.2.2 Adaptive immunity

Usually, the innate response is followed by the adaptive immune response, which consists of B and T lymphocytes. Both cell types require binding of a unique cognate antigen to the B and T cell receptor (BCR and TCR), respectively. T cells can be divided into $CD8^+$ and $CD4^+$ subsets. $CD8^+$ T cells recognise antigens loaded onto major histocompatibility complex (MHC) class I. MHC class I is expressed by virtually every nucleated cell in the mammalian body and the sensed antigens are peptides derived from cytoplasmic proteins that are degraded by the proteasome (29). In addition, exogenous proteins can be presented on MHC class I molecules of a population of DCs (30). This enables elimination of transformed cells by cytotoxic $CD8^+$ T cell (31, 32).

The $CD4^+$ T cell TCR recognises peptides presented in the context of MHC class II by antigen presenting cells (APCs) and mediate multiple functions depending on their polarization (33). On the one hand $CD4^+$ T cells can acquire a cytotoxic phenotype, but on the other hand they can differentiate into T regulatory (Treg) or T helper (Th) cells (33). The latter were initially denominated as: Th1 (producing IFN-γ and TNF), Th2 (producing IL-4, IL-5 and IL-13), Th3 (producing TGF-β) and Th17 (producing IL-17, IL-22) (33-35), but in vivo studies unveiled significant plasticity among different Th subsets (33). $CD4^+$ T cells are important for producing cytokines crucial to sustaining B as well as $CD8^+$ T cell responses, but can also limit the inflammatory reaction (33, 36). $CD4^+$ T cells provide also anti-apoptotic signalling and increase the priming capabilities APCs through CD40/CD40L interaction (37, 38).

B cells produce a broad spectrum of antibodies that can opsonize the pathogen, initiate the

complement cascade or activate effector cells in a process of antibody-dependent cell-mediated cytotoxicity (ADCC). B cells produce also cytokines and express MHC class II, and thus can serve as APCs (39, 40).
Finally, adaptive immune system mediates the memory responses. Upon recognition of a previously encountered antigen, T and B cells usually mount a faster and stronger response, which results in more efficient control of the pathogen (*18*).

1.3 Immune-mediated cancer control

The immune system can control transformed cells (*10*, *41*) and a link between leukocytes and tumour rejection has been made already in the 1940's (*42*).
The importance of the immune system is emphasized by increased incidence of mainly virus- and UV-induced cancers in immunosuppressed individuals, for example in organ transplant recipients and AIDS patients (*10, 41, 43, 44*).
Evading immune control is a crucial step in cancer progression and transformed cells developed multiple mechanisms to escape the control of immune system. This is achieved on the one hand by avoiding recognition by immune system and on the other by tumour-promoting functions of the tumour stroma.

1.3.1 NK cell-mediated tumour control

NK cells are evolutionary conserved cytotoxic lymphocytes. They express effector molecules including perforin, granzyme, TNF-related apoptosis-inducing ligand (TRAIL) and FasL (*45*) and IFN-γ (*46, 47*). Unlike $CD8^+$ T cells, their response does not depend on antigen presentation in the context of MHC class I, but rather on a fine balance between inhibitory and activating receptors. For example, NK cells are functionally inhibited by binding of killer cell immunoglobulin (Ig)-like receptors (KIRs) in humans and Ly49 in mice to MHC class I on target cells (*47, 48*). NK cells express also CD94, which recognises HLA-E in humans and Qa1 in mice. Upon forming a heterodimer with NKG2A, CD94 dampens NK cell activation (*48, 49*).
Nevertheless, loss of inhibitory ligands by target cells is not sufficient to stimulate NK cell function, and this requires additional signalling through activating receptors (*50*). For example, CD94 can also signal through NKG2C, triggering NK cell stimulation (*47, 48*). NKG2C belongs, together with NKG2D, to a C-type lectin-like receptor family, which is expressed as well by other cells of immune system (*51*). It is binding to a broad spectrum of ligands, all of which share structural homology with MHC class I proteins. In humans these

are MHC class I polypeptide-related sequence A (MIC-A), MIC-B and a group of retinoic acid early transcript 1 proteins (Raet-1) (*48, 52*). In mice it can bind to members of the retinoic acid early inducible-1 (Rae-1) protein family, minor histocompatibility antigen H60 and murine UL16-binding protein like transcript-1 (MULT-1) (*48, 52*). Ligands for NKG2D are expressed upon transformation or in stress conditions (*52, 53*). Furthermore, also genotoxic stress and activation of DNA damage checkpoint pathway drives upregulation of NKG2D ligands, an observation especially relevant in context of genetic instability of tumour cells (*54*). Cancer cells can counteract this mechanism by shedding NKG2D ligands. This has been shown in cancer patients, where elevated serum levels of MIC-A and MIC-B can serve as diagnostic markers for cancer progression (*52*). Shedding ligands not only renders tumour cells invisible to NK cells, it can also block activation of bystander NK cells, as constant stimulation with ligands leads to downregulation of the NKG2D (*52*).

Another family of activating receptors belong to the NKp family and include NKp30, NKp44, NKp46, NKp80 (*48*, *55*). There is a wide array of NKp ligands, including those encoded by viral genes and upregulation of NKp44 has been observed in NK cells infiltrating human non-small lung cancer (*56*).

A third type of activating receptors is the family signalling lymphocyte activating molecule (SLAM)-related receptors. This group includes CD226 (also known as DNAM1 or DNAX), which, similarly to NKG2D, is shared between NK cells as well as subset of $CD8^+$ T cells and myeloid cells (*48, 50, 57*). CD226 binds nectins and nectin-like molecules, like CD112 and CD155, which expression is induced by cellular stress, including DNA damage. Importantly, these ligands were found to be overexpressed in tumour cells derived from various cancers, including neuroblastoma and myeloma, where it was directly correlated with their susceptibility to NK cell attack (*50, 57, 58*). CD226-mediated activation of NK cells requires co-activation with another member of this family, the CD224 (2B4). Similar synergism was found to be necessary for NKG2D as well as NKp46 (*55*).

CD155 is a shared ligand with T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) and CD96, two inhibitory receptors expressed on both T and NK cells (*59*). Moreover, TIGIT has also additional ligands and the list of receptors controlling NK cell function is expanding, including also other members of the nectin and nectin-like protein receptors e.g. class I restricted T cell–associated molecule (CRTAM) (*59*) or CD85j (*60*).

NK cells, as well as T cells and subsets of other innate cells express also leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1). LAIR-1 binds to collagens and inhibits inflammatory response (*61, 62*). Some tumour cells hijack this mechanism and overexpress collagens, in both secreted and membrane-bound form, what is associated with increased metastatic potential (*61, 62*). Collagen can be also deposited in the tumours by

tumour-associated fibroblasts, further emphasizing the multifaceted role of stromal cells (*63*).

Aside from the canonical activating receptors, in order to recognise tumour cells, NK cells, as wells as $CD8^+$ T cells, require the adhesion molecule CD2 (*64, 65*). The ligand of CD2 is CD58, a member of the immunoglobulin superfamily. Tumour cells frequently downregulate CD58, which in context of concomitant loss of MHC class I expression, renders them insensitive to both cytotoxic T cell and NK cell surveillance (*64*).

Finally, NK cells, as well as various myeloid cells, can bind the constant region (Fc) of IgG antibodies through CD16 (FcγRIII), which enables antibody-dependent cellular cytotoxicity (ADCC) (*48, 60, 66*).

Many of the receptors and mechanisms mentioned above are shared between NK and $CD8^+$ T cells as well as other subsets of leukocytes.

Overall, NK cell interaction with other cells can be assigned into four schemes (*48*):

a) Ligation of both activating and inhibitory receptors with a healthy cell does not lead to lysis;
b) Tumour cell loses inhibitory receptors, like MHC class I, and as NK cell is being stimulated through activating receptors, tumour cell is lysed ("missing self");
c) Stress or cellular damage induces stress ligands on a tumour cell, thus overcomes the inhibitory signal and NK cell lyses the target cell ("induced self-ligands");
d) Tumour-specific antibodies are bound to CD16 on NK cell and NK cells can recognise tumour cells and trigger lysis (ADCC).

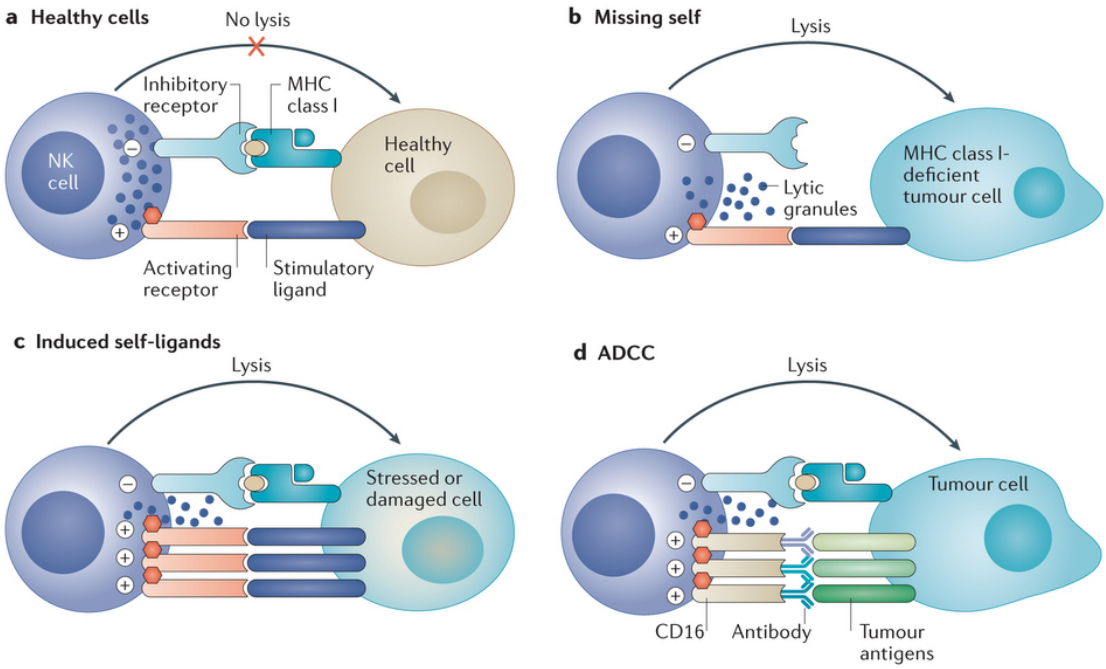

Figure 2. NK cells activity depends on a balance of activating and inhibiting signals. (**A**) NK cell interaction with healthy cell. (**B**) Tumour cells downregulate MHC class I thus cannot provide inhibitory signal. (**C**) Tumour cells upregulate stress ligands and activate NK cell. (**D**) NK cells kill tumour cell through ADCC. (Figure from: *48*)

The abovementioned schedule holds true in the context of homeostasis and an immune response against invading pathogens, whereas the situation in the context of cancer is more complex. Tumour-infiltrating NK cells are often dysfunctional and enriched in the immature, $CD56^+$ $CD16^-$ (human) or $CD27^+$ $CD11b^-$ (mouse) population, produce less IFN-γ and TNF-α and have lower cytolytic potential (*56, 60, 67*). They also express lower amounts of activating receptors NKp30, NKG2D, CD226 as well as CD16, but higher levels of inhibitory NKG2A (*60, 67*).

NK cell-dysfunction can be partially explained by the effect of tumour-derived prostaglandin E_2 (PGE_2), adenosine, indoleamine 2,3-dioxygenase (IDO), soluble ligand of FAS or transforming growth factor TGF-β. Especially the latter is a potent mediator of NK cell tolerance (*31, 48, 52*). TGF-β, PGE_2, IDO1 as well as ADAM17, a metalloproteinase involved in shedding NKG2D ligands, are overexpressed in human breast cancer (*60*). PGE_2 inhibits as well $CD8^+$ T cells, but promotes activity of $CD4^+$ $Foxp3^+$ T regulatory cells (Tregs) (*68*). Finally, many tumour-associated immune cells can inhibit the function of NK cells (*48, 69*).

NK cells survival depends on sustained Jak1/Jak3 and STAT5α/β signalling through CD122/common gamma chain (γc/CD132/IL-2Rγ) complex, also called IL-15Rβ/γ (*70*). However, IL-15 binds with a 100-fold higher activity to the trimer of IL-15Rα, IL-15Rβ and γ subunits. Macrophages and monocytes, but also fibroblastic and epithelial cell lines, broadly

express IL-15Rα, but their homeostasis is not dependent on IL-15. It has been thus suggested that IL-15Rα acts as a chaperone, increasing IL-15 half-life and enabling its trans-presentation to NK cells (*70-73*). IL-15 acts also like a chemoattractant for NK cells and stimulates their adhesion to vascular endothelium (*74*). IL-15 is also important for formation of memory $CD8^+$ T cells (*71*). Preclinical studies show that IL-15 overexpression by tumour cells supports NK cell function as well NK cell-mediated tumour control (*75*). Tumour-derived factors, like TGF-β, PGE_2 or adenosine can also affect the expression of IL-15R on NK cells (*48*).

1.3.2 $CD8^+$ T cell-mediated tumour control

$CD8^+$ T cells can eliminate cancer cells in a MHC class I-restricted response (*31*). Several studies have correlated high density of $CD8^+$ T cells infiltrating cancer lesions with beneficial prognosis in various cancer types, including breast, ovarian, colon and oesophageal cancer as well as malignant melanoma (*31, 32*). The same has been observed for cancers of viral etiology, where virus-derived peptides were presented as neo-antigens (*76*). Analogously, antigenic proteins may result from genetic mutations, potentially expanding the concept of neo-antigens to all tumours (*76, 77*). Moreover, during the complex process of dedifferentiation and acquisition of stem cell-like features, including epithelial to mesenchymal transition (EMT), tumour cells start expressing proteins and peptides which are in normal circumstances absent in the tissue, like cancer/testis antigens (*44, 78*). The mutational load directly correlates with the activity of cytolytic pathways (*44*), but T cell reactivity can be detected also against non-mutated antigens (*76*).

Tumour cells counteract recognition by $CD8^+$ T cells by downregulating the expression of MHC class I, which could explains the heterogeneous expression of MHC class I often observed in patients (*29*). MHC class I expression can be lost as a result of different mechanisms, including mutations in the proteasome (*29, 44*) or the genes coding the MHC class I loading complex (*29, 44, 79*).

1.3.3 Role of $CD4^+$ T cells in tumour progression

The role of tumour-infiltrating $CD4^+$ T cells is more ambiguous. They can perform cytotoxic functions upon recognition of an antigen in the context of MHC class II, as was shown in patients (*76, 80*). Furthermore, they can support $CD8^+$ T cell responses by secretion of type I cytokines (*80*).

However, the immunosuppressive tumour environment affects the pool of antigen presenting

cells in tumour lesion and tumour-draining lymph nodes, thus preventing $CD4^+$ T cell activation. Tumours affect maturation of lymph node DCs, as depicted by lower levels of MHC class II, CD40, CD83 and costimulatory molecules CD80 and CD86 (*60, 81*). It can be caused by elevated levels of tumour-derived IL-10 (*81*). Furthermore, vascular endothelial growth factor (VEGF) produced by tumour cells and tumour-associated macrophages (TAMs) stimulates formation of lymphatic vessels, disrupting the regular lymph flow, lymph node architecture and in the end DC migration (*81*). Moreover, the numerous population of MHC class II^+ TAMs is not inducing $CD4^+$ T cell responses due to poor antigen presentation capabilities (*21*).

The same conditions disrupt the activation of $CD8^+$ T cells rendering them anergic, but in addition the immunomodulatory tumour microenvironment skews $CD4^+$ T cells polarization into a regulatory phenotype (*35, 60, 81, 82*). Tregs are generally characterised by expression of high affinity IL-2 receptor α chain (IL-2Rα) and the fork-head transcription factor (Foxp3) (*35*). Their function has been described as immunosuppressive to both $CD8^+$ and cytotoxic $CD4^+$ T cells (*17, 35, 83*). Their presence correlates with weak anti-tumour immune response and low infiltration by $CD8^+$ T cells (*35*). In preclinical studies, selective Treg depletion was shown to be sufficient to increase $CD8^+$ T cell density in the tumour stroma (*84*).

TAMs attract Tregs through CCL-22 production, but their polarization is also induced by TGF-β and PD1/PDL1 interaction (*35, 69, 82, 85*). Furthermore, TGF-β is a potent induced of Treg proliferation (*35*). Numbers of Tregs in the lymph nodes of cancer patients normalize after tumour dissection, underlining the importance of the tumour microenvironment in the Treg differentiation (*83*).

Nevertheless, it should be noted that in contrast to murine, human $FoxP3^+$ $CD4^+$ T cells can be subdivided into immunosuppressive, so bona fide Treg population, as well as another, non-immunosuppressive population characterised by low expression of CD45RA marker. This can explain discrepancy between different studies in colorectal cancer, which have shown inconsistent correlations of Treg infiltration and cancer prognosis (*82*).

Furthermore, there are other populations of regulatory $CD4^+$ T cells, like type I T regulatory cells coexpressing CD49b and LAG-3 and producing high levels of IL-10 and TGF-β (*35, 86*). They have been involved in maintenance of peripheral tolerance (*86*) and are present in some tumour types (*35*), but their role in tumour development has not been fully delineated.

1.3.4 Tumour associated macrophages

Chronic inflammation has been recognised as one of the hallmarks of cancer and is relevant during all the stages of cancer progression (*10*). In majority of tumour types, macrophages

constitute the main cell type of immune infiltrate and their presence correlates with poor outcome of cancer patients (*87-89*). However, due to the plethora of macrophage-derived interleukins, interferons, growth factors, scavenger receptors and chemoattractant mediators, it would be an oversimplification to assign all tumour-infiltrating macrophages into a single pro- or antitumorigenic role.

Macrophages are tissue-resident phagocytes and are important in sustaining tissue homeostasis, wound healing and clearance of infections (*88*). During wound healing, tissue-resident and monocyte-derived macrophages play a unique role in tissue regeneration. As exemplified by skin repair, in the early phases of injury macrophages stimulate inflammatory response, contribute to infection control and stimulate formation of extracellular matrix and connective tissue, which is necessary for wound contraction. They produce high levels of VEGF-A, contributing to rebuilding of blood vasculature. In the later stages their phenotype is changing towards more regulatory one. They limit neutrophil activity through TGF-β production, what is necessary for prevention of tissue damage induced by neutrophil-derived proteases. TGF-β also supports homeostasis of endothelial cells and stimulates differentiation of myofibroblasts (*90-92*). This shows the plasticity of macrophages in various conditions and points that much of the protumorigenic phenotype of tumour associated macrophages (TAMs) might be mirroring the function during wound healing, referring to the description of cancer as a "wound that never heals" (*92*).

Tumours actively stimulate TAMs infiltration, from triggering production of monocytes in the bone marrow to their attraction to the tumour lesion (*93*). This is achieved by secretion of cytokines, like granulocyte- and granulocyte-macrophage-colony stimulating factor (G-CSF and GM-CSF) by the tumour cells themselves (*99, 100)*. These upregulate retinoic-acid-related orphan receptor 1 (RORC1) signalling in the bone marrow stem cells, promoting positive (like e.g. C/EBPb) and suppressing negative regulators of myeloid differentiation (like Socs3 and Bcl3). Furthermore, it imprints the commitment to the monocyte lineage by triggering IRF8 and PU.1 transcription factors (*90, 93, 94*). Such $CCR2^{+}$ monocytes migrate towards tumour-derived chemokine gradient, where they differentiate into TAMs by downregulating Ly6C and upregulating F4/80 surface markers (*95-98*). Tumours sustain macrophage differentiation and survival by production of macrophage colony stimulating factor (M-CSF, CSF1) (*90, 93, 96*).

In vitro experiments with bone marrow-derived cells have shown that depending on the stimulus, macrophages represent phenotype that can be positioned on a continuum from proficient producers of effector molecules (so-called M1, "classically activated") to a more regulatory, immune-dampening phenotype (M2, "alternatively activated" macrophages). The classically activated macrophages were initially shown to produce high amounts of IL-12, IL-1β, IL-6, TNF, as well as IL-23, ROS and NOs. On the contrary, alternatively activated

macrophages are meant to produce high level of potent immunosuppressant IL-10 and low IL-12 (*99, 100*).

Experiments in murine models show that TAMs express a mixed set of markers characteristic for both classically and alternative-activated macrophages. This includes on the one hand MHC class II, nitric oxide synthase 2 (NOS2) and inflammatory chemokines, like chemokine (C-C motif) ligand 2 (CCL2), CCL5 or interferon-inducible chemokines (C-X-C motif) ligand 9 (CXCL9), CXCL16 and CXCL10. On the other they express M2 markers like IL-10, β-glucan receptor dectin-1 and a galactose-type C-type lectin MGL1 (*95*). Others have shown that TAMs have phenotype of inflammatory CCR-2^+ Ly6C^+ monocytes that upregulated arginase I and mannose receptor CD206 in a TGF-β-dependent manner (*97*). This stands in opposition to the dichotomy proposed earlier between M1 and M2 populations (*99*).

Further studies have shown that TAMs can be divided into two transcriptionally distinct populations based on their MHC class II expression (*21, 101*) and further separated based on their Ly6C levels (*21*). Proportions between these subsets change during tumour progression, closely mirroring wound-healing and suggesting differential roles during the disease progression (*21*).

In breast cancer patients, TAMs form clusters in avascular nests of the tumour stroma (*89*). However, studies in murine models shown that TAMs penetrate both normoxic and hypoxic areas, albeit in both conditions they exhibit distinct phenotypes - MHC class II^{low} TAMs in the hypoxic nests produce more VEGF-A than the MHC class II^{high} TAMs in the normoxic parts of tumours (*21*). Formation of new blood vessels is a crucial process in the carcinogenesis as it enables overcoming the limited access to oxygen and nutrients and is referred as an "angiogenic switch" (*10, 89*). In addition to production of VEGF-A, TAMs produce matrix metalloproteinase 9 (MMP9), which further releases VEGF bound to the extracellular matrix (*102*). Others have shown have that the pro-angiogenic TAMs express as well $CD13^+$, a cell membrane-bound protease (*103*).

Tumour cells can directly drive TAMs polarization to stimulate neoangiogenesis. Hypoxic tumours switch their metabolism towards anaerobic glycolysis, which leads to accumulation of lactic acid as a by-product. Lactic acid alone is sufficient to trigger hypoxia inducible factor 1α (HIF1α) in TAMs, what orchestrates their polarization and results in VEGF-A expression (*104*). The same is achieved by tumour-derived exosomes (*24, 90*).

Furthermore, TAMs produce as well VEGF-C, a crucial molecule for remodelling of lymphatic vasculature (*85, 105*). This has profound implications in the disease progression to metastasis

As in the wound healing process, tumour growth is associated with tissue remodelling. TAMs express lysyl oxidase as well as MMP2, MMP7 and above-mentioned MMP9. Cleaving extracellular matrix releases growth factors, e.g. heparin-binding epidermal growth factor

(HB-EGF), what supports tumour progression and metastasis (*90, 98*). In addition, growth factors provided by TAMs might help tumour cells to escape apoptosis-signalling induced by anti-cancer therapy (*106*).

TAMs can also complement immune evasion through inhibition of immune reaction by suppressing the proliferation and function of T cells (*21, 97, 107, 108*). This is partially due to the effect of macrophage-derived ROS and NOs. Elevated NOs levels in the tumour microenvironment can directly nitrate TCRs, preventing antigen recognition by T cells (*107*). Furthermore, TAMs overexpress arginase I, an enzyme processing arginine, as well as IDO, which metabolises tryptophan. Both arginine and tryptophan are necessary for sustained T cell responses (*81, 108*). Furthermore, products of arginine cleavage can directly stimulate proliferation of tumour cells (*21, 100, 109*). Expression of arginase I in TAMs is induced by TGF-β and tumour-derived prostaglandins, e.g. PGE_2 (*109*).

TAMs affect T cells also by exposition of programmed death ligand 1 (PD-L1), what is sufficient to trigger T cell anergy (*110*). Moreover, they can skew $CD4^+$ T cell polarization towards tumour-beneficial Treg or Th17 phenotype by production of IL-1α, IL-1β, TGF-β and IL-6 (*34, 93, 111, 112*).

Based on these observations, it has been postulated that depletion of TAMs is sufficient to boost the efficacy of T cell activating therapies. Combination of small molecule inhibitor of CSF1R with antibody blocking PD-1 and PD-L1, reduced the tumour growth in a model of neuroblastoma (*113*). Similarly beneficial effect was observed by CSF1R inhibition and combination of immune checkpoint blockades anti-PD-1 and anti-cytotoxic T-lymphocyte-associated protein (CTLA)-4 (*114*). TAM-targeted therapies were also shown to increase $CD8^+$ T cell infiltration in mammary carcinoma models (*115*) and improved outcome of radiation therapy (*116*).

However, it should be noted that results of TAMs depletion might be model-dependent. Tumours in *Ccr2*$^{-/-}$ mice or depletion macrophages in a rat tumour model were shown to decrease infiltration of both $CD4^+$ and $CD8^+$ T cells, what negatively correlated with the survival. This is most probably a result of scarcity of TAMs-derived chemoattractants (*87, 98*).

Moreover, TAMs activated with combination of agonistic anti-CD40 antibody and IL-2 have been shown to clear tumours in a NO-dependent fashion (*117*). They can also play a protective role upon IL-12-based therapy (*118*) and be source of IL-12 and IL-18 for lymphocytes (*101, 119, 120*).

Furthermore, myeloid compartment of the tumour infiltrate contains a relatively rare population of $CD103^+$ cells, which are differentiating from monocytes and share multiple macrophage markers, but were shown to be potent activators of $CD8^+$ T cell responses and are correlated with longer survival in patients (*101*).

TAMs exhibit vast heterogeneity and high degree of plasticity. They are modulated by tumour cells and can significantly contribute to the tumour escape from immune control, but their activation can also elicit antitumoral responses.

1.4.5 Granulocytes in the tumour microenvironment

Elevated numbers of neutrophils in peripheral blood is a common observation in some cancer types (*121*). Neutrophils migrate into tumour lesions, comprising significant percentage of myeloid cells infiltrate.

Mirroring macrophage polarization, neutrophils change their phenotype during tumour development (*122, 123*). In murine transplantable models, neutrophils were shown to accumulate on the peripheries of the tumour foci early during the tumour progression, whereas they scattered among the tumour cells in the later stages. This correlates with loss of ability to attract $CD8^+$ T cells and produce TNF-α (*122*). Furthermore, neutrophils are able to inhibit NK cell function and produce metalloproteinases and IL-1β, rendering them functionally similar to protumorigenic TAMs (*124*). Some tumours stimulate expansion and release of neutrophils by expression of G-CSF, what correlates with poor prognosis (*115,124, 125*).

On the other hand neutrophils might be also relevant in the context of tumour therapy. They express FcγRs and were shown to be necessary for antibody mediated xenograft rejection in a preclinical model (*126*).

Tumour are also frequently infiltrated by eosinophils, what correlates with good prognosis in prostate, gastrointestinal, head and neck as well as bladder cancers, but at the same time with poor outcome in cervical carcinoma and Hodgkin lymphoma (*84*). Eosinophils produce CCL5, CXCL9 and CXCL10 and thus can attract $CD8^+$ T cells, but can as well secrete pro-angiogenic factors and IL-4, which stimulates the protumorigenic polarization of macrophages (*84, 90, 99*).

As depicted tumour immunome is a complex system with many cross-linked pathways involving both innate and adaptive branches of the immune system. Multiple other cell types influence the protumorigenic inflammation, just to mention ILCs, plasmacytoid dendritic cells (pDCs), γδ T cells or mast cells. Their influence vastly depends on the tumour type and stage, but they also contribute to the tumour-associated inflammation (*24, 81, 111, 127*). This is necessary for the progression towards the next step in cancer development – formation of metastasis.

1.4 The role of the immune system in metastasis

In majority of the cancer types metastatic disease is the main cause of mortality (*2, 3, 81, 124, 128*). A recent study comparing patients with and without metastasis revealed that only the lowered lymphatic vessel density and reduced expression of genes related to the immune cytotoxicity diverges the two populations (*129*), thus depicting the pivotal role of the immune system in control of the metastatic disease.

The metastatic cascade can be divided into three steps: intravasation, survival in the circulation and colonization of the distant organs. Furthermore, there is emerging evidence that tumours induce changes in distant organs prior to arrival of metastatic cells.

1.4.1 Target organs and formation of the pre-metastatic niche

Cancers often show a specific pattern of metastasis, e.g. prostate cancer to bone, colorectal cancer to the liver, and breast cancer to lung, bone and brain (*3, 130, 131*). This preferential metastases can be explained by the "seed and soil" theory, which was put forward by Stephen Paget in the late 19th century and postulates that cancer cells (the seed) changes the metastatic organ (the soil) to facilitate the tumour outgrowth. Currently, this process is often referred to as formation of the pre-metastatic niche (*3*).

Using murine transplantable models, it has been shown that tumour-derived factors like TGF-β, TNF-α, VEGF-A, serum amyloid A3 (SAA3) and alarmins S100A8/A9 diminish NK cell migration and induce inflammation in the metastasis-target organ. This correlates with accumulation of myeloid cells that facilitate tumour cell extravasation (*2, 3, 132-136*). Other studies have shown that neutrophils inhibit NK cell-mediated clearance of circulating tumour cells (CTCs) (*124*). Furthermore, accumulation of neutrophils is sufficient to attract metastasising melanoma cells, what is explained by neutrophil-stimulated inflammation and angiogenesis (*137*). Pre-metastatic niche can be also founded by immature cells, like VEGFR1^{+} bone marrow progenitors of monocytes (*138*), what is presumably the outcome of cancer-related myelopoiesis (*90, 93, 94*).

Systemic changes were also found in tumour-draining lymph nods, what included diminished proliferation of immune cells and disorganisation of T and B cell compartments. This is due to lowered CCL-21 and IL-7, but also decreased expression of chemokines for monocytes and DCs (*81, 139*). These proteins are produced by fibroblastic reticular cells as well as myeloid cells that accumulate in pre-metastatic lymph nodes and correlate with poor patient prognosis (*139-141*). Moreover, inflammatory signalling, like TNF or IL-1β induce CCL-1 expression on lymphatic endothelial cells, what can stimulate migration of CCR-8 positive cancer cells

(*142*).

The impact of protumorigenic inflammation on metastatic seeding is further illustrated by the clinical observation of cancer-to-cancer metastasis (*2, 3, 131, 143*). A whole-genome sequencing study performed on multiple metastatic foci in prostate cancer patients revealed that metastasis-to-metastasis spread is a common event (*131*). Preclinical studies have shown that this is induced by mediators of inflammation, like IL-6 and IL-8 (*144*).

It should be noted as well that multiple differences in the immune cells composition exist among different organs already in the absence of tumours. NK cells purified from different organs have varying cytotoxic capabilities (*130, 145-148*). Tissue resident macrophages and monocytes can define pattern of metastatic spread due to their differential phagocytic activity (*3, 149*). It also cannot be excluded that the majority of pre-metastatic changes occur already in the presence of CTCs or dormant metastatic cells.

There may also be anatomic reasons why particular cancer types metastasise to distinct organs (*2*). For example, liver metastasis from colorectal cancer can be explained by the blood flow through the portal vein (*130*). On the contrary, tumour-intrinsic factors, like metadherin in breast carcinomas or focal adhesion-kinase by lung carcinoma cells, can define the organ specificity (*3, 150*). Most probably the organ-targeting of metastasis is a results of anatomy, formation of a permissive niche and predisposition of tumour cells to be able to grow in a defined organ (*151*).

1.4.2 Initiation of the metastatic cascade and intravasation of tumour cells

To be able to metastasise tumour cells must obtain migratory capabilities by expressing proteases, integrin receptors, or by migration along the extracellular matrix fibres (*3, 152*). One of the theories explaining the acquisition of invasive phenotype by tumour cells is the epithelial-to-mesenchymal transition (EMT). This transdifferentiation program occurs normally during the embryonic development, but acquisition of mesenchymal traits may as well increase the motility of cancer cells (*2, 3*). This includes loss of epithelial polarization and intercellular adhesion, but also increased resistance to apoptotic signals (*2, 3, 153*). Importantly, EMT is a reversible process, as epithelial traits are beneficial in the outgrowth of a metastatic lesion (*2*). Two of the factors stimulating invasive phenotype and EMT are TGF-β and wnt-ligands derived from Tie-2^+ TAMs (*24, 60, 90, 154*), thus linking immune cells and tumour transdifferentiation.

It is a matter of discussion if EMT is sufficient and necessary to stimulate metastasis, as it was shown in a murine model that, albeit it contributed to the chemotherapy resistance, EMT is not necessary for cancer cells to colonize lungs (*155*).

Acquisition of migratory phenotype alone is insufficient for induction of metastasis, as tumour cells need to overcome blood or lymph vessel endothelium (*156, 157*). Even although the vasculature in primary tumours is considered aberrant and dysfunctional (*10*), immune cells are important controllers of tumour cell intravasation. As a matter of fact, vascular permeability occurs only in the vicinity of the protumorigenic $Tie2^{+}$ TAMs, which serve as gatekeepers for the metastasising cells. This is mediated by local production of VEGF-A and is a result of the tumour-intrinsic inflammatory reaction (*157, 158*). Similarly, TGF-β or MMP-1 produced by TAMs trigger endothelial permeability (*3*). Systemic absence of macrophages in mice lacking CSF-1 diminishes the metastatic capabilities of autochthonous mammary carcinomas (*159*). Others have shown however that targeting TAMs by CSF1R inhibition can increase the metastatic burden (*160*) and similar outcome comes from cessation of anti-CCL2 therapy, a treatment developed in order to disrupt monocyte recruitment (*161*).
Metastasis may also be promoted by inflammation triggered by conventional therapy, such as surgery, radiotherapy or even taking biopsies (*68, 162-166*).

1.4.3 Tumour cell survival in the circulation

Circulating tumour cells (CTCs) can be found in blood of patients with breast, colorectal, prostate as well as lung cancer and their increased frequency correlates with poor prognosis (*167, 168*). The typical CTC numbers vary between 1-10 cells per millilitre of peripheral blood and they have relatively short survival in the circulation, estimated at around 1-2.5 h (*2, 167, 169*). This translates into thousands of cell shed into the circulation, out of which very few progress into metastatic lesion. Thus, a very low proportion of CTC actually forms metastasis due to death in the circulation, inability to invade the target tissue or to outgrow in the parenchyma of the target organ (*153, 168*).
Upon extravasation, cancer cells encounter stress conditions, what renders them prone for attack from immune cells, especially NK cells (*3, 145, 170, 171*). NK cell-mediated CTC elimination requires close contact and there is evidence that platelets protect CTCs by shielding them (*3, 172, 173*). Thrombocytosis is often observed in cancer patients and it correlates with poor prognosis (*132*). Furthermore, association of CTC with macrophages or fibroblasts has been described (*174, 175*), and recent data showed enhanced metastatic potential of clustered CTC (*168*).

1.4.4 Colonization of the target organ

Once metastatic cells arrive in the target organ, they must extravasate and invade the

parenchyma or, in case it occluded the capillary vessel, proliferate within its bed (*2*).

For extravasation and survival CTCs require CCL-2 to both directly activate vessel endothelium and attract inflammatory monocytes (*135, 176, 177*). Monocytes directly support extravasation by local production of VEGF, analogously to the situation in the primary tumour (*178*). A transcriptome analysis of primary ovarian tumours and matched peritoneal metastases showed that CCL-2 expression was high at the metastatic sites, whereas in primary tumours this pathway was inactivated (*179*).

In some circumstances, an important role in colonization might be played also by granulocytes. Tumour cells can adhere directly to neutrophils, what connects them to the endothelium and results in increased tumour burden (*180*). Furthermore, upon stimulation neutrophils release neutrophil extracellular traps (NETs), which are webs of genomic DNA bound with bactericidal proteins, like e.g. neutrophil elastase (*181, 182*). In case of postsurgical infection, this mechanism might enable trapping CTCs and sequester them from circulation, thus facilitating establishment of metastatic lesion (*183*).

Metastatic cancer cells that escape control of immune system and overcome the growth restrictive environment of the target organ can develop clinically detectable lesions. However, some of the micrometastases do not progress for prolonged period of time.

1.4.4 Metastatic dormancy

Patients with years-long cancer remission remain under elevated risk of relapse and require monitoring (*184-186*). Outgrowth of breast cancer metastases can occur up to 20 year after initial diagnosis and removal of primary tumour, suggesting that metastases can be kept in a controlled, dormant state for prolonged periods of time (*187*). Consistently, even with frequent micrometastases in the bone marrow, only 50% patients develops any clinically detectable metastatic lesions in time frame of 10 years (*3*). Furthermore, studies on CTCs suggest that majority of the disseminated cancer cells cannot establish a lesion and are driven into a state of dormancy or undergo apoptosis (*2, 3, 132, 188*).

Dormancy might be induced by non-immune mediated mechanisms, like high production of TGF-β (*189, 190*) or anti-angiogenic factors, like angiostatin and prosaposin (*191, 192*). Alternatively, this can be explained by induction of an equilibrium state, in which the tumours persist in the organs, but are kept in check by immune cells (*41*).

First of all, clinical observations have shown that the rate of MHC class I downregulation is higher in metastatic lesions than in primary tumours (*29*). This goes in line with phylogenetically determined pattern of antigen expression in metastatic foci of various organs (*193*) and suggests that dormancy might be controlled by $CD8^+$ T cells.

Second, in preclinical studies, maintenance of dormancy of B cell lymphomas in immunized mice requires $CD8^+$ T cell-derived IFN-γ (*194*). In other models, dormancy of bone metastases is mediated by type I interferons that are produced by both T and NK cells (*187*).
Third, *RET*-oncogene transgenic mice, which develop uveal melanoma, have a prominent spread of occult cells to various organs. Depletion of $CD8^+$ T cells shortens the time between the seeding and metastasis outgrowth in lung and reproductive tract, but not skin, showing that dormancy induced by $CD8^+$ T cells might depend on the organ context (*195*).
Fourth, so-called "latency competent cancer cells", which were isolated from primary lesions of lung and breast cancer patients, can enter state of NK cell-triggered dormancy. These cells are expressing WNT inhibitor, which in an auto- and paracrine way induces downregulation of ULBP ligands of NKG2D, but also slows down cell cycling. This mechanism shows that dormancy might be also regarded as a mechanism to avoid immune surveillance (*79*).
It is important to note that cytotoxic immune cells continue their protective role even once the control over micrometastases is lost and lesions progress to clinically detectable tumours. This is exemplified by the observation that survival of metastatic melanoma patients correlates with density and composition of immune infiltrate in the metastatic lesions, mirroring observations for primary tumours (*196*).

1.5 Cancer immunotherapy

As described in the previous sections, immune cells play a profound role in control, as well support of tumour growth. It is thus an outstanding goal to design therapies that would harness patients' own immune system to fight cancer. This can be achieved by boosting the anti-cancer cytotoxic cells or by targeting the protumorigenic immune stroma. Such therapies bear the promise of treating both primary lesions as wells as disseminated tumour cells and could potentially induce anti-cancer immune memory, thus activating sustained control of tumour cells. Recent clinical trials have shown that immunotherapy is an encouraging direction in the drug development, but more work is needed to achieve successful responses in all the patients.
Several types of immunotherapies currently being evaluated, including cancer vaccines, cytokine therapies, adoptive transfers of cytotoxic lymphocytes, toll-like receptor agonists, antibodies and small molecules modifying the immunosuppressive stroma.

1.5.1 Cancer vaccines

As opposite to prophylactic vaccination, immunization against cancer necessitates activation

of immune system against an existing pathology. This requires proper adjuvants and identification of tumour-specific antigens that can be used in such preparation. Furthermore, it must overcome immune regulation, which is often associated with chronic conditions. Anti-cancer immunization protocols are currently tested in e.g. lung and skin cancers (*197*). Their development is however hindered by the scarcity of tumour antigens shared between the patients. Antigens can be also tracked individually for each patient prior to formulation of vaccine (*197*) or, the same can be achieved by adoptive transfer of patients' own APCs (*197, 198*).

The latter approach has been implemented in the only currently approved by US Food and Drug Administration (FDA) cancer vaccine, sipuleucel-T. It is a recombinant fusion protein containing prostatic acid phosphatase and GM-CSF. It is used to stimulate patient's own APCs *ex vivo*, which, upon transfer back into patient, activate lymphocytes to clear prostate cancer cells (*198*).

1.5.2 Cytokine therapies

Cytokine therapies are targeting three mechanisms: promote the APC activity, stimulate function and improve survival of the cytotoxic lymphocytes.

One of the best-studied cytokine therapies designed for the APC stimulation is based on GM-CSF. It is stimulating APC differentiation and maturation, but its overexpression in the tumour microenvironment can also trigger APC dysfunction (*96, 199, 200*). GM-CSF is tested as adjuvant for conventional therapies (*201*), but as well in anti-cancer immunization, similarly to IL-4 (*198, 202*). It can be delivered locally, by gene therapy or by transfer of non-dividing autologous or allogeneic tumour cells that express this cytokine (GVAX), what has shown beneficial effects in early clinical trials (*201, 203*).

Cytokine therapies acting directly on NK and T cells that reached clinical trials include IL-2, IL-7, IL-12, IL-15, IL-21 and type I interferons (*75, 202, 204-207*). Despite indications for clinical efficacy, their use is hindered by significant adverse side effects and short serum half-lives (*202, 208*). This can be addressed by local administration (*205*) and usage of immune complexes consisting of cytokines and anti-cytokine antibodies (*204, 207*) or cytokines and the soluble form of their receptors (*209*).

Up to date only two cytokine-based therapies have been approved for the use in clinics: IFN-α as adjuvant for advanced stage melanoma and high dose IL-2 for melanoma and renal cell carcinoma (*202, 210-212*). Although IL-2 induces durable responses in patients, the therapy with high dose IFN-α shows mixed results and patient benefits are discussable (*202, 213*).

Cytokines are currently validated as supplementary treatment to adoptive cell transfer

therapies. This includes delivery of IL-2, IL-7, IL-12 and IL-15 directly to patients, or their use for *ex vivo* expansion of lymphocytes (*202*).

1.5.3 Adoptive transfer of cytotoxic lymphocytes

Adoptive transfer of T and NK cells is tested in clinics since early 1990' and has shown promising results in therapy of blood as well as multiple types of solid tumours (*196, 214-217*). It was initially performed with autologous T cells purified from the peripheral blood or tumour. However, expansion of endogenous T cell pool is not a successful therapeutic approach due to the negative selection and dysfunction of tumour-infiltrating lymphocytes (*196, 214, 216, 217*). New technologies have been developed to improve the targeting, including transduction with genes coding antigen-specific TCRs, or chimeric antigen receptors (CARs) (*214, 216*). Whereas antigen-specific TCR enable recognition of both surface and intracellular antigens, the antigen specificity of CARs is achieved by adaptation of antibodies against tumour-specific surface molecules. This is achieved by fusion of variable region of an cancer cell-targeted antibody with and intracellular moiety derived from TCR and linked with costimulatory molecules, like CD28 or 4-1BB (*214*). These approaches are efficient in clearance of haematological malignancies, whereas treatment of solid cancers is more problematic, mainly due to necessity to achieve infiltration to the tumour stroma and then to maintain T cell function as well as avoid off-target effects (*214, 216, 218*).

The inherent requirement for successful adoptive transfer of T cells is the availability of antigen and transfer of NK cells can overcome this barrier. Anti-cancer activity is in this case achieved by partial mismatch of KIR repertoire (allogeneic transfer) or *in vitro* activation of autologous NK cells with cytokines, like IL-2, IL-12, IL-15 or IL-18 (*215, 219*). Furthermore, there are studies on possibility to use NK cell lines, what facilitates the transfer preparation (*219, 220*).

As both T and NK cells become dysfunctional in the tumour environment, it is necessary to develop novel therapies combining adoptive transfer with other immunostimulatory therapeutics, as for example cytokines (*218, 221*).

1.5.4 Blocking of immune checkpoint inhibitor pathways

One of the most successful classes of immunotherapeutics is the immune checkpoint blockade. Immune reaction depends on a balance between the stimulatory and inhibitory signalling, and blocking the latter can liberate cancer-targeted immune response (*222*). In clinics there are currently two types of checkpoint-blocking drugs – targeting cytotoxic

T-lymphocyte-associated protein (CTLA)-4 and the PD-1/PD-1L axis. Both of them have been approved for the therapy of advanced melanoma and clinical trails in other tumour types are ongoing (*223-227*)

CTLA-4 is present on both activated $CD8^+$ T cells as well as $CD4^+$ T helper cells and Tregs (*222*). The mechanism by which blocking CTLA-4 elucidates its immunostimulatory function is not fully elucidated, but might include both improved costimulatory signalling through CD28 and simultaneous depletion of Treg pool (*66, 222*). Similarly, PD-1 is initially upregulated upon TCR ligation, but then its further upregulation marks T cell exhaustion (*137-139*). PD-1 is also present on Tregs and its ligation is sufficient to induce Treg proliferation (*222*).

Furthermore, both PD-1 and CTLA-4 are present on NK cells and their blockade can increase NK cell activity, but our understanding of therapeutic role of NK cells in checkpoint blocking therapy is only emerging (*208, 228, 229*). It has been shown however that anti-PD-1 combined with anti-CTLA-4 induces NK cell-mediated protection from metastasis in a preclinical model (*208*).

Beneficial effects are also observed for treatment with anti-PD-L1 antibodies, which prevent the induction of lymphocyte anergy by blocking lymphocyte interaction with PD-L1 expressed by the tumour or stromal cells (*230, 231*).

The success of anti-CTLA-4 and anti-PD-1 turned focus into finding new targets for checkpoint blocking therapeutics. This includes multiple molecules present on T and NK cells, like lymphocyte activation gene-3 (LAG-3) (*232*), T-cell immunoglobulin mucin-3 (TIM-3) (*233*), cytokine-inducible SH2-containing protein (CIS) (*208*) and CD96 (*58*). Furthermore, there are promising results on the potential of blocking TIGIT (*234*). Upcoming clinical trials will evaluate which of these molecules are the most promising targets in cancer therapy.

Importantly, blocking multiple immune checkpoint molecules enables overcoming development of resistance and could potentially increase the response rates (*208, 233, 235*). Combination of anti-PD-1 and anti-CTLA-4 has already proven to be superior over single therapy (*223, 236*) and checkpoint blockade can also benefit from combination with other immunostimulatory therapeutics (*221*).

1.5.5 Therapy with antibodies stimulating immune response

In addition to immune checkpoint blocking antibodies, much effort has been invested in development of agonistic antibodies against immune activating receptors.

The first clinically tested antibody in this class was agonistic anti-CD28 antibody, which

activates T cells without the necessity of TCR interaction. However, the first in-human study failed due to excessive toxicity and induction of cytokine storm (*237*). This has hindered the development of antibodies against other targets, however recent studies show promising results with antibodies stimulating OX-40, CD278 and 4-1BB (*237, 238*). All of these molecules are expressed only by activated lymphocytes, reducing the risk of autoimmune reaction (*237*)

Another potential target is CD27, a molecule constitutively expressed on T and NK cells. Its ligation stimulates lymphocyte activity and it is currently tested in early clinical trials (*237, 239, 240*).

Furthermore, T and NK cells can be stimulated indirectly by targeting APCs. One of the most promising approaches is stimulation of the CD40/CD40L pathway, what in normal conditions increases antigen presentation and cytokine production, thus could potentially improve the outcome of checkpoint inhibition (*38*). Therapies based on soluble CD40L and agonistic anti-CD40 antibodies are under development and have been successfully tested in early clinical trials (*241, 242*). At the same time, further development has been hindered by strong adverse reactions related to systemic increase of inflammatory cytokines and liver toxicity (*243*). Triggering excessive systemic inflammation is an intrinsic feature of all the immunostimulatory therapies targeting receptors expressed by non-stimulated leukocytes and should be taken into consideration in designing future therapies (*237*).

1.5.6 Toll-like receptor agonists

Toll-like receptors (TLRs) sense molecules released by pathogens or own stressed and dying cells (*244, 245*). Because of their potent proinflammatory function, they have been investigated as adjuvants for vaccination as well as in cancer monotherapy.

There are currently three clinically used immunotherapeutics targeting TLRs. These are imiquimod, a TLR7 agonist approved as topical treatment of non-melanoma skin cancer (*246-248*), monophosphoryl lipid A, a lipopolysaccharide derivative as adjuvant to HPV prophylactic vaccines (*249*) and bacillus Calmettte-Guérin for treatment of non-invasive bladder cancer (*249, 250*). Furthermore, imiquimod was successfully tested in off-label therapy of lymphomas (*251, 252*). Multiple other TLR agonists are currently in clinical trials, mainly activating TLR3, TLR7 and TLR9 pathways (*253*). Resiquimod, a TLR7 and TLR8 agonist is under evaluation as adjuvants in anti-cancer peptide vaccines (*253*).

In most of the cases the direct anti-cancer mechanism of these therapeutics is not clear, but includes induction of acute local inflammation.

1.5.7 Targeting the tumour-associated myeloid cells

A quickly developing class of immune therapeutics is focusing on targeting the myeloid cells in the tumour stroma. The primal aim here is to reduce numbers of TAMs, what can be achieved by blocking migration of monocytes or their differentiation and survival.
Recent studies using small molecule inhibitors and antibodies blocking CSF1R and antibodies blocking this receptor showed promising results in preclinical models (*106, 114, 116, 254, 255*). Clinical trials to determine clinical applicability of such and approach are currently ongoing, as CSF1R blockade is tested in solid tumours, in a single treatment regimen and in combination with checkpoint blocking antibodies (*113, 256*). The first available results for antibody blocking CSF1R showed no dose limiting toxicities, but further studies are necessary to determine efficacy of such therapy (*256*).
On the other hand, preclinical studies have shown that pharmacological inhibition of monocyte migration by blocking CCL-2 can have adverse effects on the metastatic outcome (*161*). Targeting TAMs with the currently available drugs inherently targets all the monocytes and it can have profound systemic side effects.

1.6 Models of metastasis

Our understanding of the biology and therapy of metastatic disease is hindered by the scarcity of biologically relevant models.
Even though new models are being developed, much of our knowledge is based on models of experimental metastasis. Here tumour cells are injected intravenously into the tail vein, giving rise to "metastatic" lesions in the lungs. In addition, intrasplenic injection of tumour cells followed by splenectomy results in "metastases" in the liver and intracardiac injection results in spread to bones (*257, 258*). Although these models enable studying metastatic seeding, they cannot address other events of the metastatic cascade including the impact of the primary tumour. Furthermore, a single injection of a large number of tumour cells (usually in hundreds of thousands) does not compare with continuous shedding of low numbers from the primary tumour.
Another approach is to use xenograft models, in some of which animals develop spontaneous metastases. However, these models don't allow studying the interaction of metastasising cancer cells with the adaptive immune system, thus have limited applicability in cancer immunology (*259, 260*). New generations of humanized mice might partially overcome these problems. Alternatively, induction of central immune tolerance might be achieved by microinjection of tumour cells into the blastocysts of immunocompetent mice (*260*).

The metastatic cascade is best studied in mouse models of breast cancer. For example, the autochthonous PyMT carcinoma model, in which carcinogenesis is triggered by expression of the polyoma virus middle T protein. These mice develop mammary hyperplasia at four weeks of age, which progresses to carcinoma and form variable number of metastatic lesions, mainly in the lung (*261*). This is however a lengthy process and tumour bearing mice have to be kept until old age. Other metastasising breast carcinoma model is the injection of the 4T1 cells orthotopically in the mammary fat pad, what gives rise to prominent lung metastasis in a reproducible way and a relatively short time frame (*262*).

Orthotopic models have been developed also for colorectal cancer. Injection of tumour cells in the colon and cecum enables establishment of primary lesions that give rise to liver metastases (*263*).

Other autochthonous models include RET.AAD mice (expression of human oncogene *RET* and chimeric mouse/human MHC antigen AAD), which develop uveal melanoma that in older animals metastasises to multiple organs, including lungs, brain and reproductive track (*195*). Also TRAMP (transgenic adenocarcinoma of the mouse prostate) mice progress to metastatic disease, albeit mainly to the lymph nodes (*264*). The drawbacks of most spontaneous models are that it takes long until metastases occur, they are not synchronised and incidence is often low.

Resection of subcutaneous tumours in immunocompetent mice is an approach is to study all the aspects of metastasis. This is the setup I have used in my experiments.

2. Aims

The aim of my thesis was to contribute to our understanding of how components of the immune system impact on metastatic seeding or progression. Therefore, I have established a model of spontaneous metastasis from subcutaneously injected Lewis Lung Carcinoma cells (LLC, 3LL) that I have tagged with luciferase using lentiviral transduction. In this model 50-60% of mice develop metastasis mainly to lung, liver and draining lymph nodes, which allowed the analysis of interventions that either promote or prevent metastasis.

I have addressed following questions:

1. Inhibition of signalling via the CSF1R aims to deplete tumour-promoting macrophages and is currently in clinical trials. Preclinical data suggest that CSF1R-blockade inhibits the growth of primary tumours, however, very little data are available on the impact of CSF1R-blockade on metastasis. **I have investigated the effect of CSF1R-blockade on metastatic seeding and progression**.
2. Recent data have shown that production of the pro-inflammatory mediator PGE_2 in the tumour promotes tumour growth, mainly by inhibiting immune defence. As the role of tumour-derived PGE_2 on metastasis is largely unknown, **I have compared metastasis formation from resected tumours that do or do not produce PGE_2**.
3. It is well established that NK cells are important in controlling metastatic seeding of tumour cells and recent data suggest that, in analogy to T cells, the function of NK cells is under checkpoint control. Therefore, **I have investigated whether immune stimulation by agonistic and/or checkpoint-blocking antibodies impact on the rate of metastasis**.
4. ALCAM is an adhesion molecules expressed by a wide variety of cells and, amongst others, plays a role in leukocyte transmigration through blood and lymphatic vessels. Because intra- and extravasation are crucial steps in the metastatic cascade, **I have investigated whether ALCAM contributes to development of spontaneous metastasis.**

3. Results

3.1 CSF1R-dependent monocytes are required for NK cell-mediated control of metastasis

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Introduction

Despite recent advances in oncology, metastatic disease remains the major cause of cancer-related death. The cascade of events involved in this process includes invasion of surrounding tissue, intravasation into the blood or lymph vasculature, survival in the circulation and extravasation and colonization of distant tissue. Each of these steps involves crosstalk between tumor cells and hematopoietic cells, including lymphocytes and different cell types of myeloid origin (*1*).

NK cells were primarily recognized to remove circulating tumor cells (*2, 3*) and CD8+ T cells can control transformed cells in the primary tumors as well as in the metastatic lesions (*4, 5*). Whereas CD8+ T cells became an attractive target for immune-activating therapies (*6, 7*) and approaches to boost NK cell responses are under development (*8, 9*), the development of therapeutic strategies based on targeting the myeloid cell compartment have proven more complicated due to the dual role these cells play in the tumor progression and metastasis. On the one hand, myeloid cells can produce proinflammatory cytokines and survival factors for NK and T cells such as IL-2, IL-12 and IL-15 (*10-14*) and comprise antigen-presenting cells (APCs) that are crucial to T cell activation (*15*). On the other hand, they are recruited to tumors where they contribute to local immunosuppression, promote tumor growth and directly facilitate formation of metastases (*16-19*). Targeting tumor-associated macrophages (TAMs) by inhibition of CSF1R has shown promising results in preclinical cancer models (*20-23*). However, little is known about the impact of such treatment on the formation or progression of metastasis.

Here we aimed to diminish metastatic disease by manipulating the myeloid compartment using two interventions that are currently being evaluated in clinical trials: Agonistic anti-CD40 antibody (*24*) and blockade of CSF1R (*25*), thus activating APCs and depleting TAMs, respectively.

Results

Anti-CD40 protects against metastatic disease

We used a model of spontaneous metastasis from resected, primary Lewis lung carcinoma tumors expressing luciferase (LLC-LUC) in immunocompetent mice. Approximately 50% of mice developed metastases preferentially in the lungs, livers and tumor-draining lymph nodes within 3 weeks after surgery. In order to investigate whether monotherapy with anti-CD40 prevents seeding of metastatic cells or their progression in the metastatic organ, we started treatment before (Fig. 1A, upper panel) or after the surgery (Fig. 1A, lower panel), respectively. Administration of anti-CD40 shortly before resection reduced the rate of metastasis by 50-70%, whereas administration immediately after resection had no impact on the metastatic frequency or load (Fig. 1B). The metastatic burden of those mice that developed metastases despite anti-CD40 treatment before surgery was comparable to that of untreated mice (Fig. 1C and 1D). This suggests that the control of metastasis triggered by anti-CD40 occurs early in the metastatic cascade, e.g. during the seeding phase. We confirmed the protective activity of anti-CD40 treatment in a model of spontaneous metastasis from breast cancer tumors (4T1) in BALB/c mice (Fig. S1).

Inflammatory response to anti-CD40 causes a favorable switch in Treg to CD8+ T Cell ratio, but the anti-metastatic effect is independent of T Cells

To understand how anti-CD40 protects against metastasis, we first measured the amount of proinflammatory cytokines in the serum 24 h after injection of anti-CD40. Anti-CD40 triggered the production of interferon g (IFNg), tumor necrosis factor α (TNFα), interleukin (IL)-12 and IL-6 (Fig. 2A), pointing towards a type I immune response signature (*26*). In addition, we detected increased amounts of the monocyte chemoattractant chemokine (C-C) motif ligand 2 (CCL-2) (*27*) (Fig. 2A).

Because anti-CD40 was effective only when given before resection of the primary tumor, we analyzed immune populations in the primary tumor immediately after resection. Treatment with anti-CD40 did not change the number of neutrophils, inflammatory monocytes, macrophages, NK or CD8+ T cells (Fig. S2), but led to a IFNg-dependent (*28*) and data not shown) deletion of tumor-infiltrating $CD4^+$ T cells (Fig. 2B). Although this decrease was not specific to T regulatory cells (Tregs) (Fig. 2C and S2), it resulted in a favorable decrease of the Tregs to $CD8^+$ T cell ratio (Fig. 2D) (*29, 30*).

Based on these results, we reasoned that the beneficial effect of anti-CD40 required sufficient tumor antigen, activated APCs and T cells. As the number of CD4+ T cells was diminished upon anti-CD40 treatment, we assumed that CD8+ T cells were the main effector cells preventing metastasis. We depleted CD8+ T cells 24 h prior to anti-CD40 treatment (Fig. 2E), which resulted in sustained depletion until the time point of resection (Fig. S3). Absence of $CD8^+$ T cells did not, however, influence the size of the primary tumor (Fig. S4) and had no impact on the anti-CD40-mediated reduction of the metastatic burden (Fig. 2F). Thus, anti-CD40 protects against metastases independently of T cells.

Anti-CD40 triggers systemic activation of NK cells and NK-mediated clearance of metastasizing tumor cells

We observed increased amounts of IL-12 in the serum after anti-CD40 treatment (Fig. 2D). Because IL-12 is a potent activator of NK cells (*31*), we analyzed the phenotype and function of NK cells in lungs of tumor-bearing mice, as the lungs are most frequently affected by metastasis in this model. Twenty-four hours after anti-CD40 treatment NK cells produced (*2*) and upregulated the expression of molecules associated with NK cell activation and effector function, including killer cell lectin-like receptor subfamily G member 1 (KLRG1), NKG2D, Fas ligand (FasL) and granzyme B (Fig. 3A), in an IL-12-dependent fashion (Fig. S5) (*10, 32, 33*). Furthermore, anti-CD40 induced systemic changes in the maturation of NK cells, as measured by changes in the surface levels of CD11b and CD27 (*34*), with an accumulation of $CD11b^-$ $CD27^+$ cells (Fig. 3B).

To investigate whether NK cells are essential for the anti-metastatic effect of anti-CD40, we depleted NK cells starting immediately prior to anti-CD40 treatment and continuing until tumor resection (Fig. 3C). Depletion of NK cells had no effect on the size of primary tumors (Fig. S6), however, it resulted in a dramatically increased rate of metastasis (Fig. 3D), suggesting that NK cells do not act on the primary tumor but rather control metastasis in the circulation and/or at the metastatic site. Depletion of NK cells completely prevented the protective effect of anti-CD40 treatment (Fig. 3D), indicating that anti-CD40-mediated protection against metastasis essentially depends on NK cells.

Because the effect of anti-CD40 did not require the activity of T cells, we considered it unlikely that antigen supply is a major function of the primary tumor in this context.

Alternatively, the primary tumor may serve as continuous supply of circulating, metastasizing tumor cells, which have a short half-life in the circulation (*35, 36*), and anti-CD40 may prevent metastasis at the level of circulating tumor cells. To directly address whether NK cells are involved in the elimination of circulating LLC-LUC tumor cells, we injected tumor cells intravenously (i.v.) (Fig. 3E). Depletion of NK cells prior to i.v. injection resulted in increased tumor burden in the lungs and reduced survival of mice (Fig. 3F and data not shown).

Targeting tumor-associated macrophages abolishes the protective effect of anti-CD40 and promotes metastatic disease

Macrophages, defined as CD45.2+ CD11b+ Ly6G- F4/80+ Ly6Cint CSF1R+ cells (Fig. S2A, S7), are numerous in LLC-LUC tumors (Fig. S2) and are often described to play a tumor-promoting role (*37*). Therefore, we argued that targeting TAMs may potentiate the anti-metastatic effect of anti-CD40. To address the impact of TAMs on the development of metastasis, we administered BLZ945, a small-molecule inhibitor that blocks CSF1R signaling (CSF1Ri), and thus prevents the development and maintenance of various myeloid cell subsets including TAMs (*23, 38, 39*). We administered CSF1Ri starting 3 days before the first dose of anti-CD40 and continuing until resection (Fig. 4A). Administration of CSF1Ri strongly reduced the numbers of TAMs (Fig. 4B), but had no impact on the growth of primary tumors (Fig. 4C). Instead of the expected synergy between anti-CD40 and CSF1Ri, we observed that CSF1Ri abolished the anti-metastatic effect of anti-CD40 (Fig. 4D). We next investigated the impact of TAM-reduction by CSF1Ri on the rate of metastasis independently of anti-CD40 and treated mice with CSF1Ri from the day of injection of tumors until surgery (Fig. 4E). Also in this situation, CSF1Ri resulted in an increased rate of metastasis (Fig. 4F) without impact on primary tumor growth (Fig. S8). When CSF1Ri was given immediately after the primary tumor was resected, the metastatic rate was unchanged (Fig. 4F and 4G). We confirmed these observations using antibody-mediated blockade of CSF1R in the 4T1 model of spontaneous metastasis from breast cancer in BALB/c mice, thus excluding the possibility that our results are a peculiarity of the small molecule inhibitor of CSF1R, surgery, the mouse strain or the model used (Fig. 5A, 5B and 5C).

Systemic inhibition of CSF1R affects NK cell homeostasis

To understand why CSF1R blockade promoted metastasis and prevented the anti-metastatic effect of anti-CD40, we determined circulating leukocyte counts in mice treated with CSF1Ri. As expected, treatment with CSF1Ri for 3 days reduced numbers of Ly6Chi and Ly6Clow monocytes (Fig. 6A), whereas numbers of CD4+, CD8+ and B cells as well as neutrophils did not change (data not shown). However, treatment with CSF1Ri decreased numbers of NK cells (Fig. 6B), which is explained by the fact that NK cell homeostasis depends on IL-15 trans-presented by CSF1R-dependent monocytes (*40*).

Administration of exogenous IL-15 restores NK cell numbers and allows metastasis control under CSF1Ri treatment

To address whether increased metastasis after CSF1Ri was caused by the concomitant reduction of myeloid cells or of NK cells, we treated mice with CSF1Ri and supplemented one group with IL-15/IL-15Rα complexes (IL-15c) in order to rescue NK cell numbers in the face of low numbers of CSF1R-dependent myeloid cells (*41, 42*) (Fig. 6C). CSF1Ri-treated mice showed strongly reduced NK and myeloid cell

counts, whereas CSF1Ri-treated mice receiving exogenous IL-15c showed normal NK but still low myeloid cell numbers (Fig. 6D and 6E, and data not shown). Decreased numbers of NK cells but not myeloid cells correlated with increased metastatic load in the lungs (Fig. 6F and 6G). Together these data demonstrate that NK cells are essential for the protection against metastatic seeding to the lungs. Although IL-15 is also required for survival of CD8+ memory T cells (*43*), we do not think that the effects described here involve CD8+ T cells, as depletion of this subset had no impact on metastasis formation in the model of spontaneous metastasis.

Discussion

There is increasing evidence that metastasis is under control of the immune system (*44-46*). Using clinically relevant models of spontaneous metastasis formation, we have investigated whether modulating the myeloid compartment impacts on the rate of metastasis. Specifically, we used anti-CD40 to activate APCs and CSF1R-blockade to target tumor-promoting TAMs in pre- and post-surgical regimens. Both of these approaches are currently tested in clinical trials (*24, 25*).

We demonstrated that activation of the CD40 pathway before resection of the primary tumor protects against metastasis independently of T cells. Our observations that those mice developing metastases despite anti-CD40 treatment have a comparable metastatic burden to the control cohort and treatment is not successful when primary tumor is resected suggest that anti-CD40 interferes with the seeding of metastatic cells, rather than with progression of metastatic lesions. We identified NK cells as the major protective population against spontaneous metastasis and found that anti-CD40 activates NK cells at different anatomical sites in an IL-12-dependent fashion. This goes in line with a recently published phase I study of a chimeric anti-CD40 agonistic antibody, in which both elevated levels of serum IL-12 and peripheral NK cell activation was observed (*24*). Although NK cells are essential to control metastasis, we observed little or no influence of these cells on the progression of the primary tumor. The fact that primary tumors contain few infiltrating NK cells may explain this observation, but the compromised NK cell maturation within tumors may also be important (*47, 48*). In addition, circulating tumor cells may be particularly vulnerable to NK cells, as stress conditions such as oxidative or shear stress can upregulate the expression of ligands for NKG2D on tumor cells (*49, 50*).

Our attempt to further potentiating anti-CD40 treatment by depletion of tumor-promoting TAMs, however, unexpectedly nullified anti-CD40-mediated protection. This stands in apparent contrast to published findings showing that CSF1R-blockade retarded the development of primary tumors (*20, 23, 51*), but goes in line with the observation that CSF1R-blockade promotes the development of metastases (*52*). Swierczak et al. showed that CSF1R-blockade increased the amount of G-CSF in the serum and therefore speculated (but did not show) that neutrophilia is the main cause for increased metastasis. Neutrophilia downstream of CSF1R-blockade, however, may be a peculiarity of tumors that produce high amounts of G-CSF such as 4T1 (*3, 53*) as we do not observe it in BLZ945-treated mice. Furthermore, it was recently shown that anti-macrophage therapy based on blocking C-C chemokine ligand 2 (CCL-2) increases the risk to develop metastasis upon treatment cessation due to enhanced angiogenesis and monocyte counts (*54*). Thus, targeting myeloid cells in cancer may be less straightforward than thought and a better understanding of the role that different myeloid subsets play in various aspects of cancer-related immunity is urgently needed.

We show that depletion of TAMs and other CSF1R-dependent myeloid cells concomitantly reduces the number of NK cells, thus providing a mechanistic explanation of the apparently controversial action of CSF1R-blockade (*20, 23, 51, 52*). Maintenance of peripheral NK cells depends crucially on IL-15, as it controls the intracellular levels of anti-apoptotic Bcl-2 (*55*). NK cells express IL-15Rb/g, which binds IL-15 with high affinity only when it is trans-presented on IL-15Ra (*56*) expressed by myeloid cells in steady state conditions (*40*). We observed that the detrimental effect of CSF1R-blockade on metastasis is reversed by exogenous IL-15/IL15-Ra complexes (*41, 42*), which restores the number of NK cells in the face of low numbers of CSF1R-dependent myeloid cells. Together, these data show that NK cells are essential for protection against metastatic seeding and at the same time excludes a major role for myeloid cells other that supporting NK cell survival. This may seem to contradict the recently published findings by Hanna et al., who showed that control of metastasis required the presence of Nr4a1+ patrolling monocytes (*45*). Nr4a1+ patrolling monocytes are Ly6Clow monocytes (*57*) that are enriched in the microvasculature of the lung. Although this study showed that Nr4a1+ patrolling monocytes attract NK cells, a functional role of NK cells in protection against metastasis was not addressed. As patrolling monocytes express CSF1R (*45*), they may be targeted by CSF1Ri as well. We did not specifically investigate Nr4a1+ monocytes here, but the fact that supplementation with IL-15/IL15-Ra complexes rescues NK cell counts without restoring the numbers of myeloid cells and prevents CSF1Ri-promoted metastasis, clearly identifies NK cells as the major cell type protecting against metastasis to the lung. NK cells are currently being investigated as potential effector cells in multiple cancer therapy approaches, including in vivo cytokine therapies (*58, 59*) and adoptive transfers (*8, 9*) thus opening additional beneficial perspectives in a clinical application of such a treatment.

In summary, we found that anti-CD40 prevents the development of metastatic disease in an NK cell-dependent fashion. Moreover, our results suggest that NK cells control the seeding of circulating tumor cells to the metastatic site rather than progression of metastasis. The fact that control of metastasis crucially depends on NK cells also explains why blockade of CSF1R signaling promoted metastasis: CSF1Ri-mediated reduction of myeloid cells concomitantly diminishes the amount of IL-15 that is trans-presented to NK cells and is essential for their survival. This is of clinical relevance because of ongoing clinical studies on CSF1R-targeting therapies.

Thus, although blockade of CSF1R-signaling depletes TAMs and may impede tumor progression, at the same time it may foster metastatic disease, suggesting that this intervention should be applied under particular circumstances and with great care. Our results suggest that treatment with CSF1Ri or CSF1R-blocking antibodies targeting TAMs bears the risk of increasing metastatic disease, which can be efficiently counteracted by concomitant administration of an NK cell survival factor such as IL-15.

Materials and Methods

Mice

C57BL/6JOlaHsd1 mice were obtained from Harlan Laboratories (Envigo), *Il12rb2*$^{-/-}$ mice from Jackson and BALB/cJRj mice from Janvier. Mice were kept under specific pathogen-free conditions at the Laboratory Animal Services Center at the University of Zurich.

Female mice of 6-8 weeks were used for all experiments. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by The Cantonal Veterinary Office Zurich.

Cell lines

LLC1 cells were obtained form ATCC and lentivirally transduced to express firefly luciferase (LUC) generating LLC-LUC cells. Viral particles were a gift from Prof. Christian Münz (University of Zurich). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% with fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin (all Invitrogen). 4T1 cells were a gift from Prof. Michael Detmar (Swiss Federal Institute of Technology in Zurich) and were grown in Roswell Park Memorial Institute (RPMI) 1640 Medium supplemented with 10% FBS, 2 mM L-glutamine, penicillin and streptomycin (all Invitrogen)

Models of metastasis

For spontaneous metastasis from LLC-LUC tumors, C57BL/6 mice were injected subcutaneously (s.c.) with 2x105 LLC-LUC cells in 100 µl PBS. Groups were randomized before the start of treatment. After 25-28 days mice were anesthetized with 2.5% attane (Piramal Healthcare Ltd.) and 0.04 mg/kg fentanyl (Kantonsapotheke Zurich) injected intraperitoneally (i.p). Primary tumors were subsequently resected and wounds were clipped with Autoclip wound clips (Becton Dickinson). Mice with primary tumors below 200 mg were removed from the analysis of the metastatic rate. For post-operative analgesia, temgesic (Schering-Plough) was given s.c. at 0.1 mg/kg immediately after surgery and in drinking water at 10 µg/ml for 48 hours ad libitum. Three weeks after surgery mice were anesthetized with 2.5% attane (Piramal Healthcare Ltd.) and injected i.p. with 150 mg/kg D-luciferin (TBD-Biodiscovery). Photon flux in vivo as well as from dissected organs was measured using an IVIS 200 imaging system (Perkin Elmer). To compare data between experiments, rates of metastasis of treatment groups were normalized to the rate of the control group, which was set at 100%.

For spontaneous metastasis from orthotopic breast cancer, 1x105 4T1 cells in 50 µl PBS were injected into the second mammary fat pad of BALB/c mice and animals were sacrificed on day 23 or 25, as indicated. Mice with primary tumors below 1500 mg were removed from the analysis of metastatic burden. For counting metastases, India ink (15% in PBS) was injected intratracheally, lungs were removed, washed with PBS and fixed in Fekete's solution (62% ethanol, 3.3% formaldehyde, 0.25 M acetic acid). Metastatic foci were counted blinded using a dissecting microscope.

For experimental metastasis, C57BL/6 mice were injected intravenously (i.v.) into the tail vein with 5x105 LLC-LUC cells in 200 µl PBS. Mice were sacrificed on days mentioned in the Fig. legends and bioluminescence was measured as described above.

Treatment of animals

Mice were injected with 50 µg anti-CD40 (FGK45) or isotype antibody (2A3) per dose. To deplete CD8+ T cells, 500 µg anti-CD8 (YTS169.4) was injected at day -1 relative to the first dose of anti-CD40. To deplete NK cells, 200 µg anti-NK1.1 (PK136) was injected at day -2 and day 0 relative to anti-CD40 in the spontaneous model or on day -3 and -1 relative to i.v. injection of LLC-LUC tumor cells. Anti-CD40, anti-NK1.1 and anti-CD8 antibodies were purified from hybridoma culture supernatant using protein G sepharose 4 Fast Flow (Sigma-Aldrich). Isotype antibody was obtained from BioXCell. Anti-CSF1R antibody (AFS98) was obtained from

BioXCell and was used at 500 µg per dose. All antibodies were administered i.p. in 200 µl PBS.

The small molecule inhibitor of CSF1R (CSF1Ri) BLZ945 was supplied by Novartis AG and dissolved at 12.5 mg/ml in 20% Captisol as vehicle (Ligand). CSF1Ri or vehicle were administered daily per os (p.o.) at 200 mg/kg.

IL-15 complexes were prepared by mixing recombinant murine IL-15 (Affymetrix eBioscience) and recombinant murine IL-15Ra (R&D Biosystems) in a 1:1 molar ratio and subsequent incubation at 37°C for 30 minutes as described (29). IL-15 complexes were administered i.p. at 4.5 µg/mouse in 200 µl of PBS.

Measurement of inflammatory cytokines

Serum was collected using vacutainer rapid serum tubes (BD) and inflammatory cytokines were quantified using a CBA Mouse Inflammation Kit (BD).

Flow cytometry

Primary tumors were collected in PBS, cut into pieces and digested for 45 minutes at 37°C in RPMI medium containing 10% FBS, 1 mg/ml collagenase IV and 2.6 µg/ml DNase I (both Sigma-Aldrich). Samples were washed with PBS by centrifugation for 5 minutes at 350 g, the pellet was resuspended in PBS and filtered to remove debris. For intracellular IFNγ detection cells were incubated for 4 hours at 37°C in presence of 10 µg/ml brefeldin A (Sigma-Aldrich). For surface staining antibodies against the following proteins were used: CD3 (17A2), CD4 (RM4-5), CD8a (53-6.7), CD11b (M1/70), CD11c (N418), CD27 (LG.3A10), CSF1R (AFS98), CD45.2 (104), CD49b (DX5), F4/80 (BM8), FasL (MFL3), Granzyme B (NGZB), KLRG1 (26), Ly6C (HK1.4), Ly6G (1A8), MHCII (M5/114), NK1.1 (PK136), NKp46 (29A1.4). For viability staining, Zombie Violet Fixable Viability Kit was used. Antibodies and viability stain were purchased from Biolegend, except for Granzyme B (Affymetrix eBioscience). For surface and viability staining, samples were incubated in PBS for 25 minutes at 4°C. Subsequently, cells were washed and fixed for 25 minutes with 4% paraformaldehyde in PBS. For intracellular staining, samples were washed with permeabilization buffer (PBS containing 20 mM EDTA, 2% FBS, 0.03% NaN3, 0.1% saponin) and incubated overnight with antibody against IFNγ (XMG1.2) (Biolegend). FoxP3 staining was performed according to the manufacturer's protocol (Affymetrix eBioscience). For quantitative analysis, CountBright absolute counting beads were used (Thermo Fisher Scientific). Samples were acquired using a CyAn ADP 9 flow cytometer (Beckman Coulter) and analyzed using FlowJo v9.8.5 software (Tree Star).

Histology

Five µm thick sections of formalin-fixed, paraffin-embedded tissue were stained with hematoxylin (Merck Millipore) and eosin (Carl Roth AG) according to a standard protocol. Pictures were taken using a Leica DMI 6000 microscope.

Statistical analysis

For comparison of two experimental groups, two-tailed Student's t-test with Welch's correction was performed. More than two groups were compared using non-parametric Kruskal-Wallis tests with Dunns post-tests. Rates of metastases were compared using chi-square test. All tests were performed with GraphPad Prism 5.0 and GraphPad QuickCalcs (GraphPad Software). * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

Supplementary Figures

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Acknowledgments

We thank Anne Müller, Alexander Knuth, Lubor Borsig and Christian Münz for valuable input and support. We thank the personnel from the Laboratory Animal Service Center (University of Zurich) and Rodent Center HCI (ETH Zurich) for expert animal care. This work was financially supported by the Swiss National Science Foundation (SNSF), Oncosuisse and the University Research Priority Program (URPP) “Translational Cancer Research”. James Sutton is an employee of Novartis International AG.

Figures

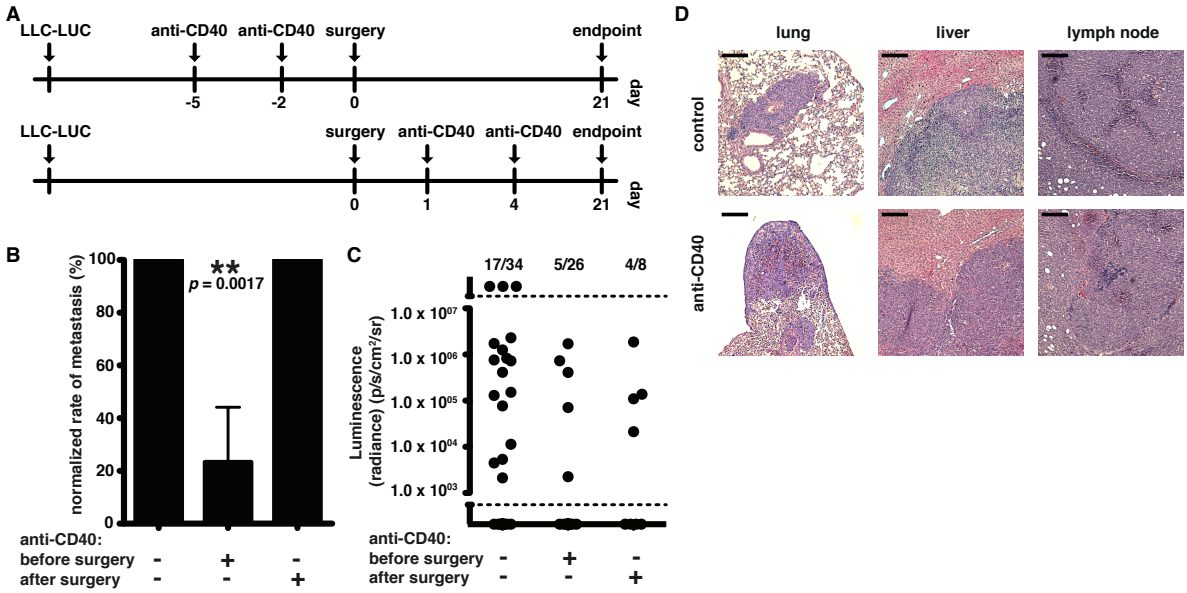

Figure 1. **Administration of anti-CD40 reduces metastasis from resected tumors.** (**A**) Experimental timelines. (**B**) Rate of metastasis normalized to the control group. Mean ± SD. **p<0.005 (Chi-square test). Groups consisted of 5-9 mice. Representative data from at least 3 independent experiments are shown. (**C**) Metastatic burden measured as cumulative luminescence signal from lungs, tumor-draining lymph nodes and livers in mice with metastatic lesions from (B). Points above the upper dotted line depict mice that had to be sacrificed before the endpoint because of high metastatic burden. Points below the lower dotted line depict mice that were metastasis-free. Each symbol represents an individual mouse. (**D**) Representative sections of metastatic lesions in lungs from data shown in (B) stained with haematoxylin and eosin. Scale bar 200 μm.

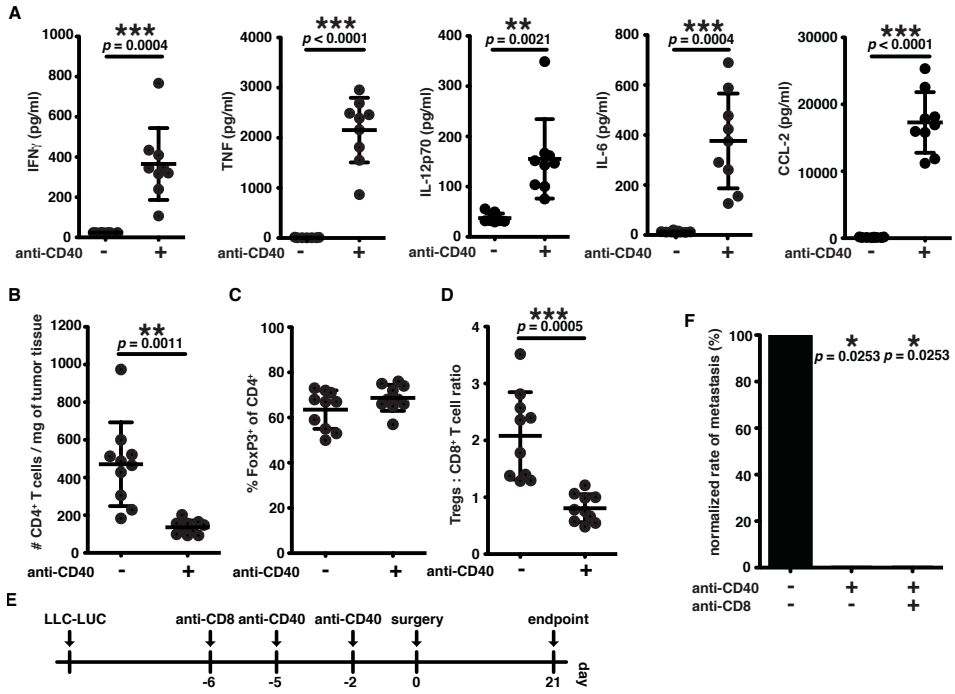

Figure 2. **Anti-CD40 triggers a type I immune response, but controls metastasis independently of CD8+ T cells.** (**A**) Amount of inflammatory cytokines in the serum 24 h after the first dose of anti-CD40. Each symbol represents an individual mouse. Mean ± SD. **p<0.005 ***p<0.0005 (two-tailed Student's t-test with Welch's correction). (**B-D**) Flow cytometry analysis of the LLC-LUC tumor infiltrate on the day of surgery (d 28). Each symbol represents an individual mouse. Mean ± SD. **p<0.005 ***p<0.0005 (two-tailed Student's t-test with Welch's correction). (**E**) Treatment schedule. (**F**) Rate of metastasis normalized to the control group. *p<0.05 (Chi-square test). Groups consisted of 5 mice.

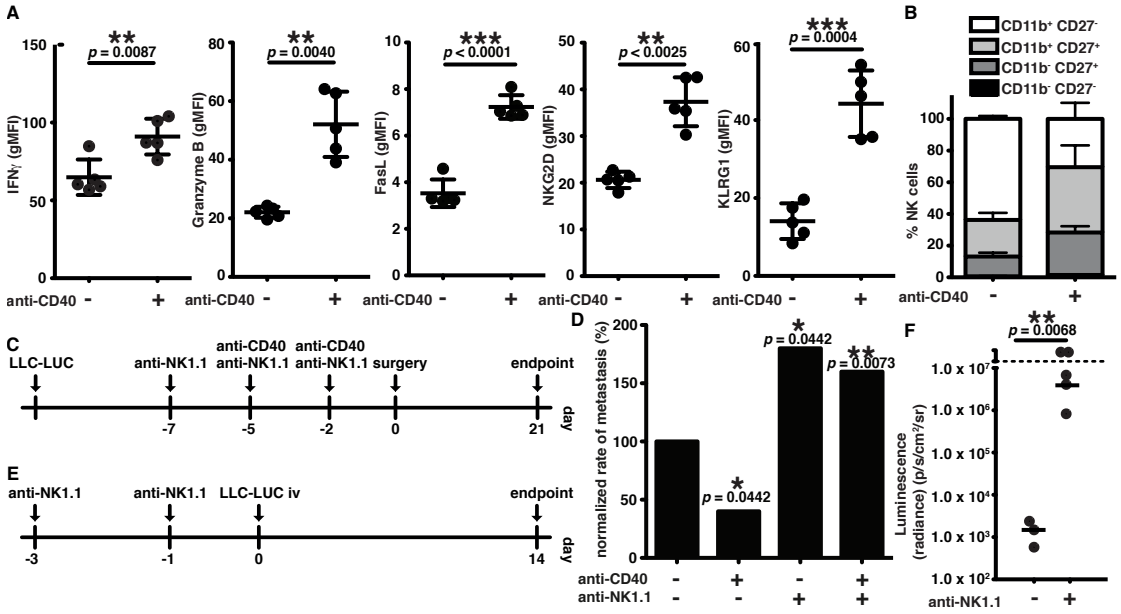

Figure 3. **Anti-CD40 activates NK cells that are crucial to control of metastasis.** (**A**) Expression of IFNγ, Granzyme B, FasL, NKG2D and KLRG1 by lung NK cells in tumor-bearing mice measured by flow cytometry 24 hours after injection of anti-CD40. Each symbol represents an individual mouse. Mean ± SD. ***p<0.0005 (two-tailed Student's t-test with Welch's correction). Representative data from at least 2 independent experiments are shown. gMFI - geometric mean fluorescence intensity. (**B**) NK cell maturation in the spleens of tumor-bearing mice measured by flow cytometry 24 hours after injection of anti-CD40. Pooled data from 3 mice. Mean ± SD. (**C**) Experimental timeline. (**D**) Rate of metastasis normalized to the control group. *p<0.05 **p<0.005 (Chi-square test). Groups consisted of 9-10 mice. Representative data from 3 independent experiments are shown. (**E**) Experimental timeline. (**F**) Tumor burden in lungs quantified as luminescence signal of lungs. Points above the upper dotted line depict mice that had to be sacrificed before the endpoint because of high metastatic burden. Each symbol represents an individual mouse. Mean ± SD. **p<0.005 (two-tailed Student's t-test with Welch's correction).

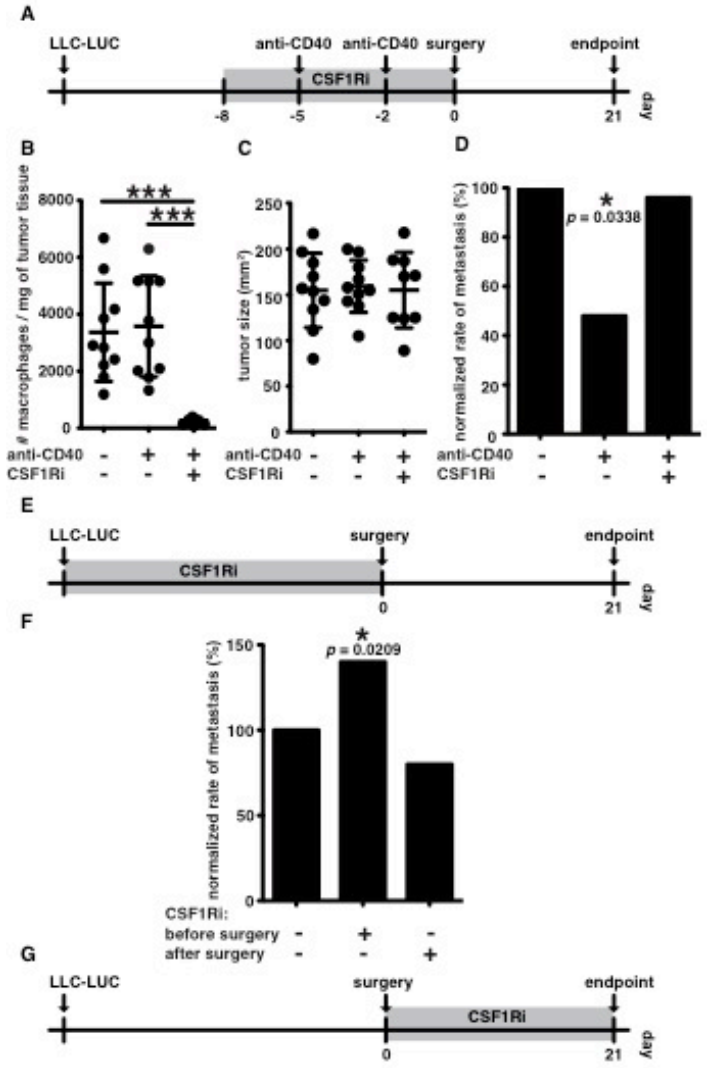

Figure 4. **CSF1R blockade abrogates the anti-metastatic effect of anti-CD40 and increases the rate of metastasis.** (**A**) Experimental timeline. (**B**) Flow cytometry analysis of the LLC-LUC tumor infiltrate on the day of surgery (d 27). Each symbol represents an individual mouse. Mean ± SD. ***p<0.0005 (non-parametric Kruskal-Wallis tests with Dunn's post-tests). Representative data from 2 independent experiments are shown. (**C**) Tumor sizes on the day of surgery. Each symbol represents an individual mouse. Mean ± SD. ***p<0.0005 (non-parametric Kruskal-Wallis tests with Dunn's post-tests). Representative data from 2 independent experiments are shown. (**D**) Rate of metastasis normalized to the control group. *p<0.05 (Chi-square test). Groups consisted of 10 mice. Representative data from 2 independent experiments are shown. (**E**) Experimental timeline. (**F**) Rate of metastasis normalized to the control group. *p<0.05 (Chi-square test). Groups consisted of 4-7 mice. Representative data from 2 independent experiments are shown. (**G**) Experimental timeline.

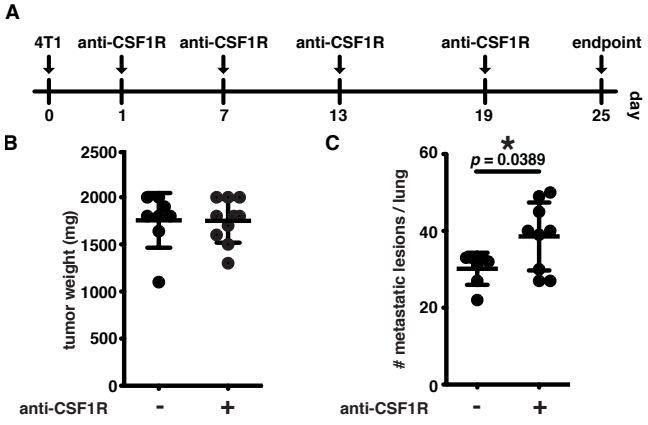

Figure 5. **Anti-CSF1R treatment promotes metastasis from orthotopic breast cancer.** (**A**) Experimental timeline. (**B**) Tumor weight on the endpoint. Mean ± SD. (**C**) Number of lung metastases. Mean ± SD. *p<0.05 (two-tailed Student's t-test with Welch's correction). Each symbol represents an individual mouse. Representative data from 2 independent experiments are shown.

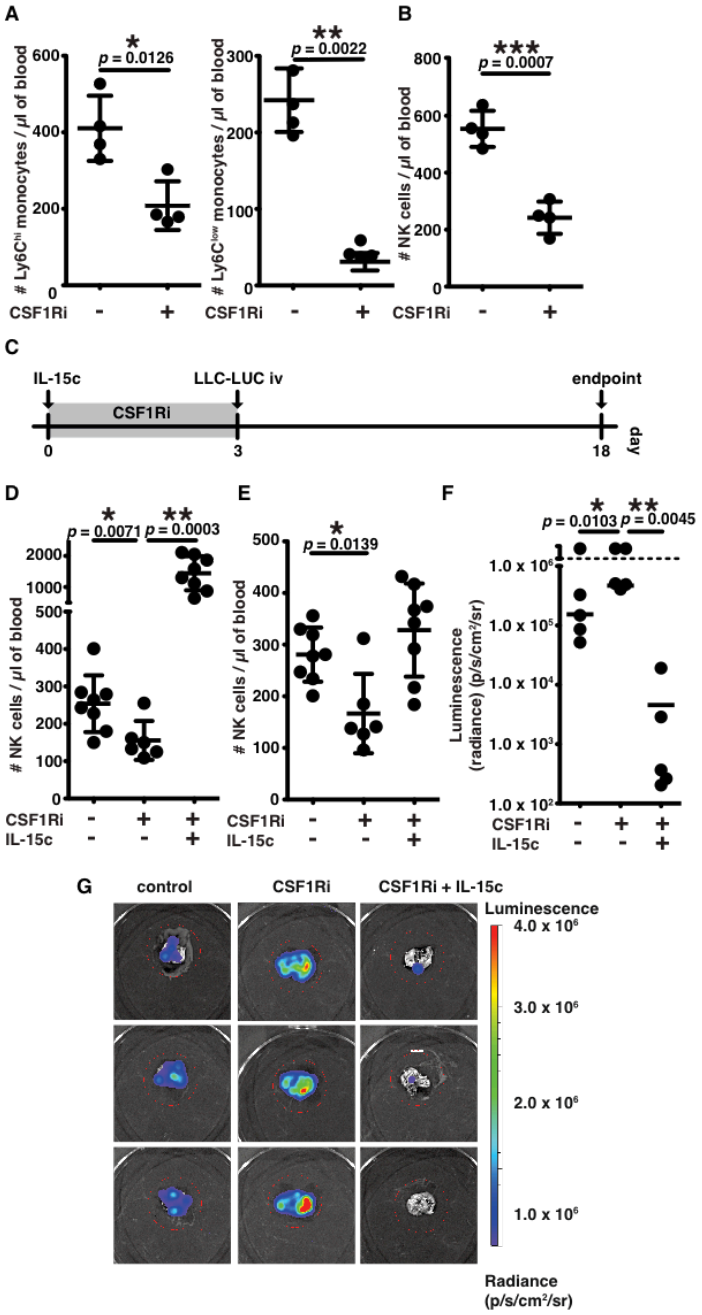

Figure 6. **Increase of metastasis mediated by CSF1R blockade is caused by disrupted NK homeostasis and can be restored by supplementation with IL-15.** (**A**) Numbers of inflammatory (Ly6Chi) and patrolling (Ly6Clow) monocytes in blood measured by flow cytometry 3 days after the start of CSF1Ri treatment. Each symbol represents an individual mouse. Mean ± SD. *p<0.05 **p<0.005 ***p<0.0005 (two-tailed Student's t-test with Welch's correction). Representative data from 2 independent experiments are shown. (**B**) Numbers of NK cells in blood measured by flow cytometry 3 days after the start of CSF1Ri treatment. Each symbol represents an individual mouse. Mean ± SD. *p<0.05 **p<0.005 ***p<0.0005 (two-tailed Student's t-test with Welch's correction). Representative data from 2 independent experiments are shown. (**C**) Experimental timeline. (**D**) Numbers of NK cells in circulation on day 3 and (L) day 7 after the start of treatment. Each symbol

represents an individual mouse. Mean ± SD. *p<0.05 **p<0.005 (two-tailed Student's t-test with Welch's correction). Representative data from 2 independent experiments are shown. (**E**) Quantification of tumor burden in the lungs. Points above the upper dotted line depict mice that had to be sacrificed before the endpoint because of high metastatic burden. Each symbol represents an individual mouse. Mean ± SD. *p<0.05 **p<0.005 (two-tailed Student's t-test with Welch's correction). Representative data from 2 independent experiments are shown. (**F**) Representative bioluminescence images of the lungs.

Supplementary Figures:

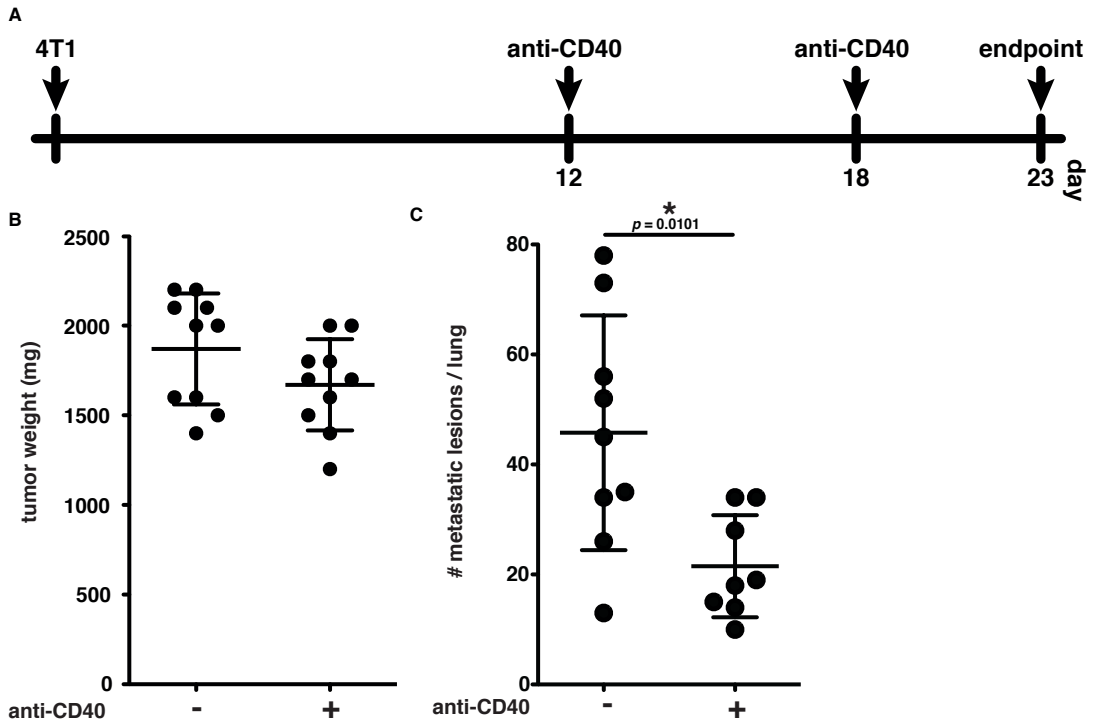

Figure S1. **Anti-CD40 reduces metastases from orthotopic breast cancer.** (**A**) Experimental timelines. (**B**) Tumor weight at the endpoint. Mean ± SD. (**C**) Number of lung metastases. Mean ± SD. *p<0.05 (two-tailed Student's t-test with Welch's correction). Each symbol represents an individual mouse. Representative data from 2 independent experiments are shown.

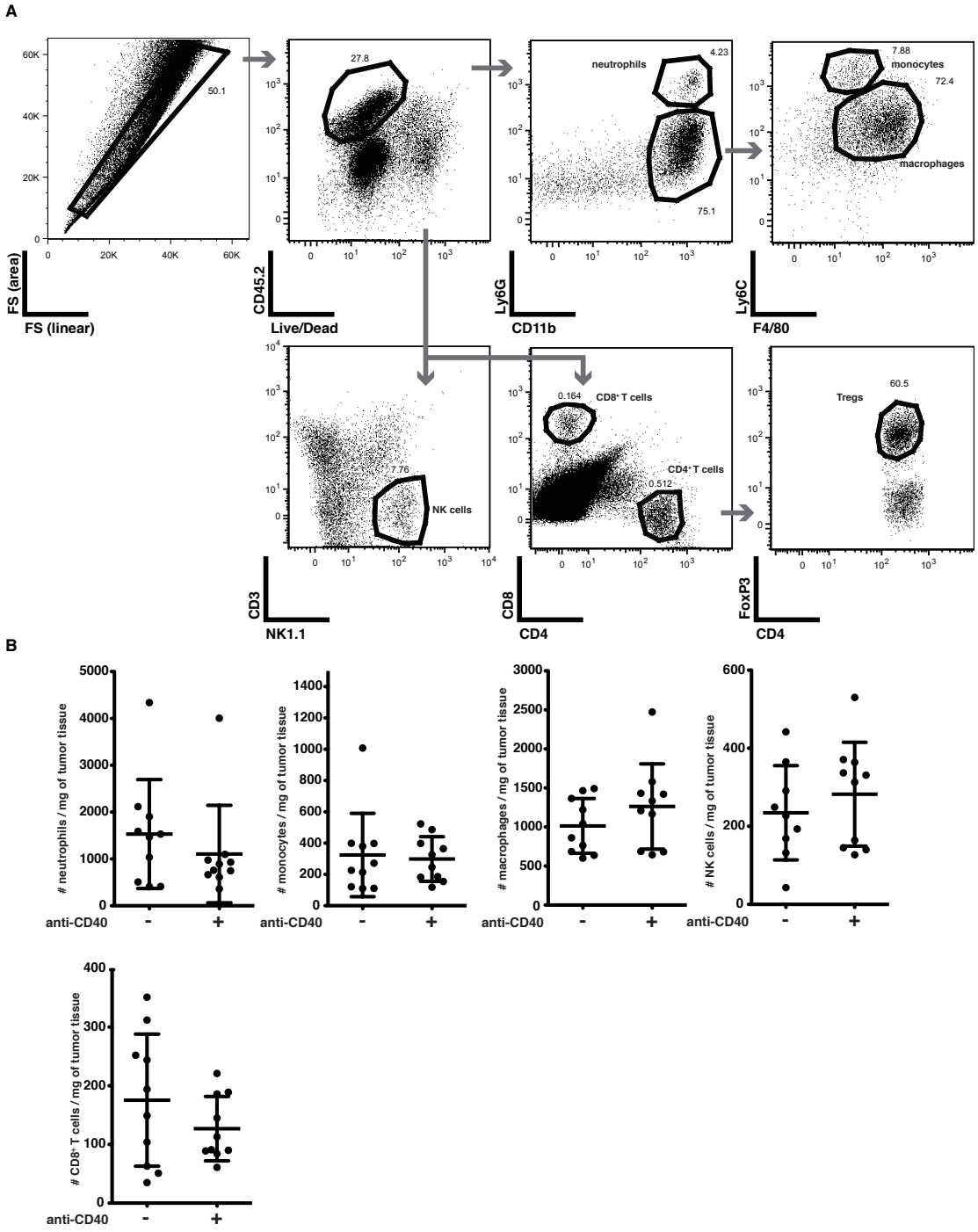

Figure S2. **Immune infiltrate of primary LLC-LUC tumors.** (**A**) Gating strategy. (**B**) Flow cytometry analysis on the day of surgery (d 28). Mean ± SD. Each symbol represents an individual mouse. Representative data from 5 independent experiments are shown.

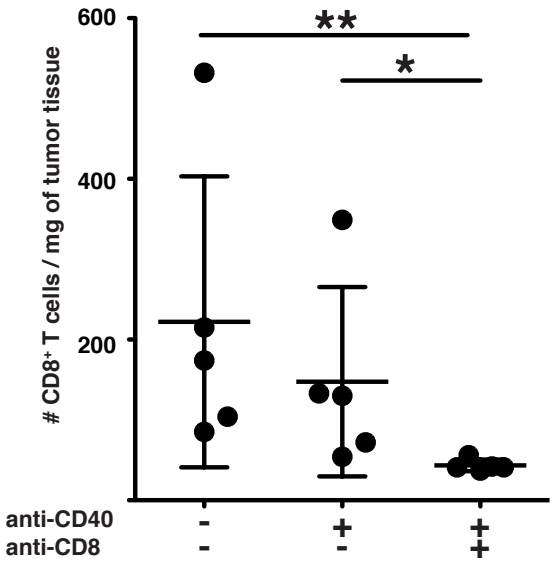

Figure S3. **Depletion of CD8+ T cells.** Flow cytometry analysis on the day of surgery (d 31). Mean ± SD. *p<0.05 **p<0.005 (non-parametric Kruskal-Wallis tests with Dunn's post-tests). Mice were treated as described in Figure 2E. Each symbol represents an individual mouse.

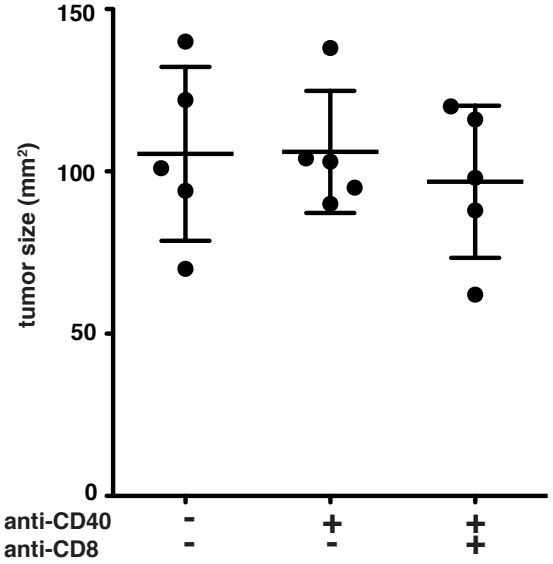

Figure S4. **Depletion of CD8+ T cells has no impact on the size of primary LLC-LUC tumors**. Measurement taken on the day of surgery (d 31). Mean ± SD. Mice were treated as described in Figure 3C. Each symbol represents an individual mouse.

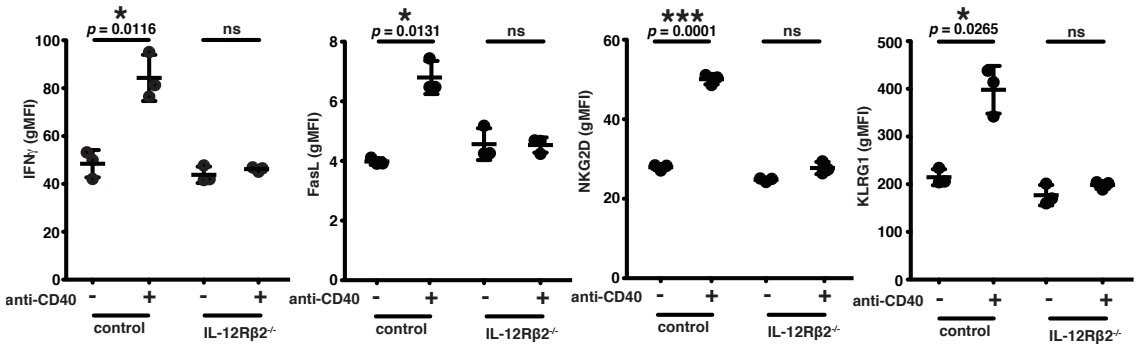

Figure S5. **Anti-CD40 activates NK cells in an IL-12-dependent manner.** Expression of IFNγ, FasL, NKG2D, KLRG1 by lung NK cells measured by flow cytometry 24 hours after injection of anti-CD40 in wild-type (control) and *Il12rb2* deficient mice (IL-12Rb2-/-). Each symbol represents an individual mouse. Mean ± SD. *p<0.05 ***p<0.0005 (two-tailed Student's t-test with Welch's correction). ns – not significant. gMFI - geometric mean fluorescence intensity.

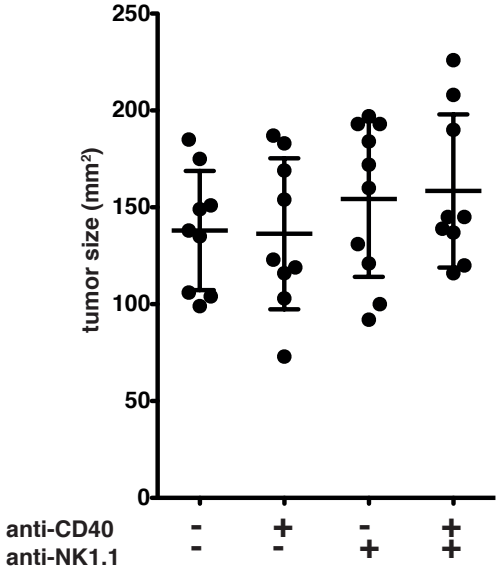

Figure S6. **Depletion of NK cells with anti-NK1.1 antibody cells has no impact on the sizes of primary LLC-LUC tumors.** Measurement taken on the day of surgery. Mean ± SD. Mice were treated as described in Figure 3C. Each symbol represents an individual mouse. Representative data from 3 independent experiments are shown.

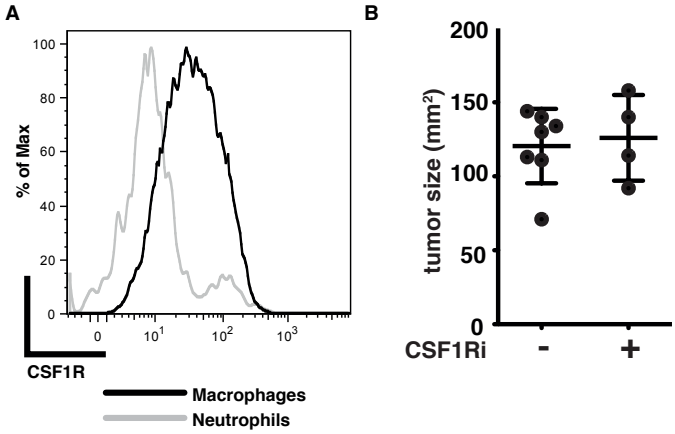

Figure S7. **Targeting CSF1R-expressing TAMs has no impact on the size of primary LLC-LUC tumors.** (**A**) Flow cytometry analysis. A representative example of 5 mice is shown. (**B**) Size of primary tumor measured on the day of surgery (d 21). Mean ± SD. Mice were treated as described in Figure 4A. Each symbol represents an individual mouse. Representative data from 2 independent experiments are shown.

3.2 The role of tumour-derived prostaglandin E_2 in the development of metastatic disease

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3.2.1 Introduction

Prostaglandin E_2 (PGE_2) is an important mediator of inflammation. It is produced by myeloid cells, like macrophages and neutrophils (*100, 265*) and triggers vascular permeability and fever, thus playing a significant role in the early steps of inflammatory response (*266*). However, recent studies have shown that tumours hijack this mechanism to induce tumour-promoting chronic inflammation (*267*) and render T and NK cell dysfunctional (*60, 267, 268*). PGE_2 can also induce production of IL-23 by dendritic cells (DCs), which drives differentiation of $CD4^+$ T cells into tumour-beneficial IL-17-producing cells (*269*). Furthermore, PGE_2 can stimulate macrophage polarization towards a tumour-promoting phenotype, which induces neovasculogenesis through vascular endothelial growth factor (VEGF) production. Besides this, PGE_2 induces as well VEGF-independent angiogenesis (*161, 268*).

PGE_2 synthesis is a three-step process, which starts with the release of arachidonic acid from the cell membrane and processing into PGG_2 and PGH_2 by cyclooxygenase (COX)-1 and COX-2. PGH_2 is further metabolised into various prostaglandins by respective enzymes. PGE_2 production depends on three independent synthases, namely membrane-bound PGE synthase (mPGES)-1, mPGES-2 and cytosolic PGES (cPGES). These enzymes have different subcellular localization (mPGES-1 in the perinuclear membrane, mPGES-2 in both Golgi and cytosol whereas cPGES in the cytosol only) but also a different preference for the upstream COX enzymes: mPGES-1 processes PGH_2 from COX-2, mPGES-2 from COX-1 and COX-2, whereas cPGES mainly from COX-1 (*270*).

COX-2 has been associated with intestinal tumorigenesis (*271*) and its overexpression has been observed in tumour-associated macrophages (TAMs) (*272*). COX-2 inhibitors potentiate anti-angiogenic therapy in preclinical studies (*268*) and are tested for colorectal cancer treatment (*273*), exemplifying the importance of prostaglandin-mediated inflammatory mechanisms in the cancer development or progression

We aimed to investigate whether PGE_2 has an impact on the metastatic cascade. We used a model of spontaneous metastasis from resected Lewis Lung Carcinoma (LLC) tumours, which produce high amounts of PGE_2 (*109, 274, 275*). This has been correlated with tumour cell motility (*275*), ability to establish tumours after subcutaneous injection into syngeneic

mice (*274*), but also induction of immunosuppressive function in tumour-associated as well as alveolar macrophages (*109, 276*). It remains however unclear if PGE_2 is directly involved in the process of metastasis.

Here we show that disruption of PGE_2 production in cancer cells is sufficient to reduce their ability to metastasise, which is correlated with increased infiltration of the primary tumours with $CD4^+$ T and NK cells, but decreased numbers of neutrophils.

3.2.2 Materials and methods

Mice

C57BL/6JOlaHsd1 mice were obtained from Harlan Laboratories (Envigo). Mice were kept under specific pathogen-free conditions at the Laboratory Animal Services Center at the University of Zurich.

Female mice of 6-8 weeks were used for all experiments. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by The Cantonal Veterinary Office Zurich.

Model of spontaneous metastasis

Mice were injected subcutaneously (s.c.) with 2×10^5 LLC-LUC or LLC-LUC-mP1/mP2/$cP^{-/-}$ cells in 100 µl PBS. Groups were randomized before the start of treatment. After 25-28 days mice were anesthetized with 2.5% attane (Piramal Healthcare Ltd.) and 0.04 mg/kg fentanyl (Kantonsapotheke Zurich) injected intraperitoneally (i.p). Primary tumours were subsequently resected and wounds were clipped with Autoclip wound clips (Becton Dickinson). Mice with primary tumours below 200 mg were removed from the analysis of the metastatic rate. For post-operative analgesia, temgesic (Schering-Plough) was given s.c. at 0.1 mg/kg immediately after surgery and in drinking water at 10 µg/ml for 48 hours ad libitum. Three weeks after surgery mice were anesthetized with 2.5% attane (Piramal Healthcare Ltd.) and injected i.p. with 150 mg/kg D-luciferin (TBD-Biodiscovery). Photon flux in vivo as well as from dissected organs was measured using an IVIS 200 imaging system (Perkin Elmer). To compare data between experiments, rates of metastasis of treatment groups were normalized to the rate of the control group, which was set at 100%.

Cell lines

Parental LLC-LUC tumour cells are described in chapter 3.1. To generate PGE_2-deficient LLC-LUC cells, we used the microbial clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system to mutate three downstream PGE synthases:

mPGES-1, mPGES-2 and cPGES (*270, 277*). To maximise chances of disrupting their enzymatic function, we aimed to introduce double strand breaks in the vicinity of the active centres of the enzymes, which were arginine^{100} in mPGES-1 (*278*), cysteine^{110} (*279*) and tyrosine^{9} in cPGES (*280*). Single-guide RNA (sgRNA) were designed using an online tool (*281*) and cloned into a plasmid coding CRISPR backbone as well as Cas9 tagged through a self-cleaving AA peptide with enhanced green fluorescent protein (eGFP). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% with fetal bovine serum (FBS), 2 mM L-glutamine, penicillin and streptomycin (all Invitrogen).
Transfected cells were sorted based on their GFP expression using FACS Aria III 5L (Becton Dickinson). After the last sorting round, we have derived monoclonal cell lines.

Plasmid purification and cloning

The backbone plasmid pSpCas9(BB)-2A-GFP (Addgene) was purified from *Escherichia coli* culture using QIAGEN Plasmid Kits (Qiagen), digested using BbsI restriction enzyme (Thermo Fisher Scientific) and purified by gel electrophoresis using QIAquick Gel Extraction Kit (Qiagen). Ligation of sgRNA into the backbone was achieved using T4 DNA Ligase (NEB).

sgRNA design, synthesis and DNA sequencing

sgRNA were designed using CHOPCHOP tool (*281*). Synthesis of sgRNA and sequencing of DNA was performed by Microsynth AG.

Transfection

LLC-LUC cells were transfected using branched Mw=25000 polythylenimine (PEI) (Sigma Aldrich). Briefly, 10^6 LLC-LUC cells were seeded on a single well of 6 well plate. 2 µg of plasmid was mixed with 15 µg of PEI in FBS-free medium and incubated for 8 minutes in room temperature. Cells were washed with PBS. Plasmid-PEI solution was mixed with complete medium and added to the cells for 2 h. After that time, cells were washed and medium was changed to standard. Transfection efficacy was checked by flow cytometry 24 h after washing.

Cell sorting

Transfected cells were sorted based on their GFP expression using FACS Aria III 5L (Becton Dickinson).

Flow cytometry

Primary tumours were collected in PBS, cut into pieces and digested for 45 minutes at 37°C

in RPMI medium containing 10% FBS, 1 mg/ml collagenase IV and 2.6 µg/ml DNase I (both Sigma-Aldrich). Samples were washed with PBS by centrifugation for 5 minutes at 350 g, the pellet was resuspended in PBS and filtered to remove debris. For surface staining antibodies against the following proteins were used: CD3 (17A2), CD4 (RM4-5), CD11b (M1/70), CD19 (6D5), CD45.2 (104), F4/80 (BM8), Ly6C (HK1.4), Ly6G (1A8), NK1.1 (PK136). For viability staining, Zombie Violet Fixable Viability Kit was used. Antibodies and viability stain were purchased from Biolegend. For surface and viability staining, samples were incubated in PBS for 25 minutes at 4°C. For quantitative analysis, CountBright absolute counting beads were used (Thermo Fisher Scientific). Samples were acquired using a CyAn ADP 9 flow cytometer (Beckman Coulter) and analyzed using FlowJo v9.8.5 software (Tree Star).

Quantitative Real Time PCR

RNA was purified using TRI Reagent following producer's protocol (Ambion). Remaining DNA was digested using 1 unit DNase I (NEB) for 10 µg of RNA. Retrotranscription was performed using MultiScribe Reverse Transcriptase following producer's protocol (Thermo Fisher Scientific). Real Time PCR was performed using EvaGreen mastermix (Biotum) and LightCycler 480 thermocycler (Roche Life Science). Transcript levels were normalised to *Gapdh* as an internal control. Following primers were used:

- *Gapdh*

FWD 5'-TCGTGGATCTGACGTGCCGCCTG-3'

REV5'-CACCACCCTGTTGCTGTAGCCGTAT-3'

- *mPGES-1*

FWD 5'-GCACACTGCTGGTCATCAAG-3'

REV 5'-ACGTTTCAGCGCATCCTC-3'

- *mPGES-2*

FWD 5'-CGTGAGAAGGACTGAGATCAAA-3'

REV 5'-GAGGAGTCATTGAGCTGTTGC-3'

- *cPGES*

FWD 5'-CGAATTTTGACCGTTTCTCTG-3',

REV 5'-TGAATCATCATCTGCTCCATCT-3'.

mPGES-1, *mPGES-2* and *cPGES* primers were designed using Universal ProbeLibrary Assay Design Center tool (Roche Life Science).

PGE_2 detection

Cells were seeded at 100 000 cells / well on a 96-well plate. After 24 h supernatants were collected and PGE_2 was quantified using PGE_2 ELISA kit (Enzo Life Sciences).

Statistical analysis

For comparison of two experimental groups, two-tailed Student's t-test with Welch's correction was performed. Rates of metastases were compared using chi-square test. All tests were performed with GraphPad Prism 5.0 and GraphPad QuickCalcs (GraphPad Software). *p<0.05, **p<0.005, ***p<0.0005.

3.2.3 Results

Generation of PGE_2-deficient cell lines

Transfection of LLC-LUC using polythylenimine resulted in a low albeit reproducible transfection rate (<5%) and cells retained their GFP expression for up to 4 days (data not shown). First we have performed transfection with a plasmid encoding sgRNA for *mPGES-1* and sorted GFP^+ cells. Second, we have repeated the procedure with a plasmid encoding the *mPGES-2* sgRNA and finally with *cPGES* sgRNA. After the last sorting round, we have derived monoclonal cell lines. Further selection has been done based on the amount of transcripts of the three synthases (Fig. 1 A) and production of PGE_2 (Fig. 1 B) resulting in selection of a cell line with the desired phenotype (LLC-LUC-mP1/mP2/$cP^{-/-}$).

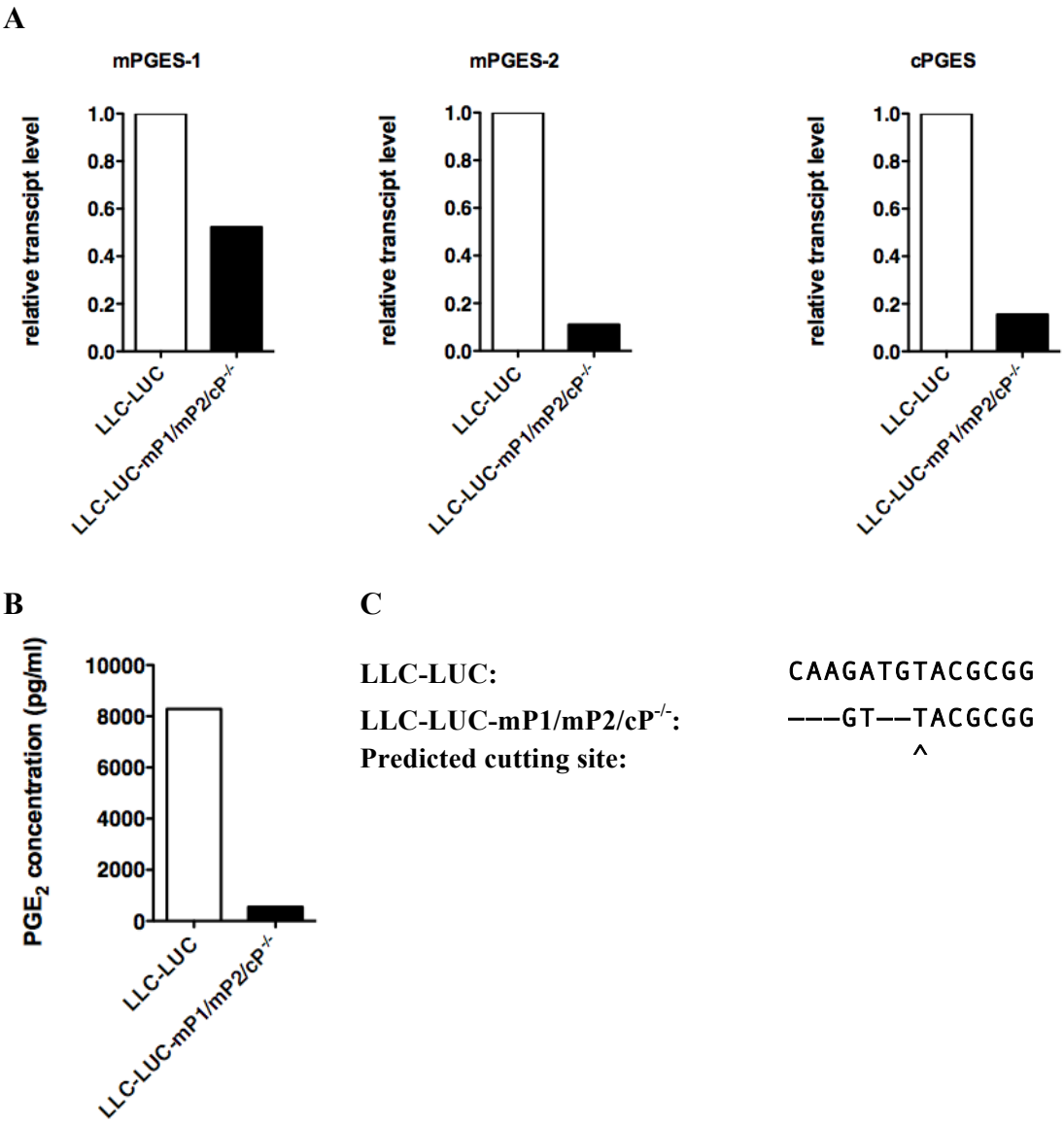


Figure 1. Generation of LLC-LUC derivative deficient in PGE_2 production (**A**) Amounts of *mPGES-1*, *mPGES-2* and *cPGES* transcripts measured by quantitative real time PCR. (**B**) PGE_2 measured by ELISA. (**C**) Double strand break induced in the *mPGES-1* gene introduced a two-nucleotide deletion. Sequencing of the genomic DNA of parental and LLC-LUC-mP1/mP2/$cP^{-/-}$.

There is no quantitative difference in *mPGES-1* transcripts between the parental LLC-LUC and LLC-LUC-mP1/mP2/$cP^{-/-}$, but targeted sequencing of *mPGES-1* around the predicted cutting site revealed introduction of mutations (Fig. 1 C).

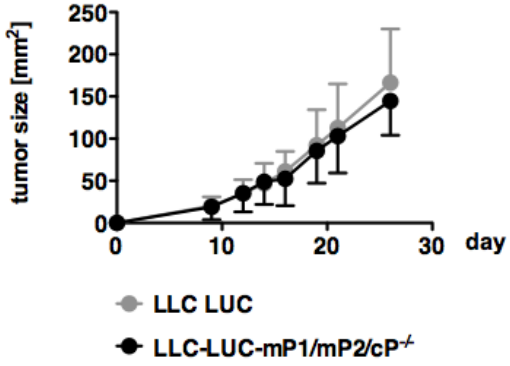
These data confirm that we have successfully generated a cell line deficient in synthesis of PGE_2.

Reduction of tumour-derived PGE_2 does not influence the growth of primary tumours, but induces changes in their immune infiltrate

To validate whether tumour-derived PGE_2 is important in the metastatic cascade, we have

injected both parental and LLC-LUC-mP1/mP2/cP$^{-/-}$ cells subcutaneously into C57BL/6 mice.

A



B

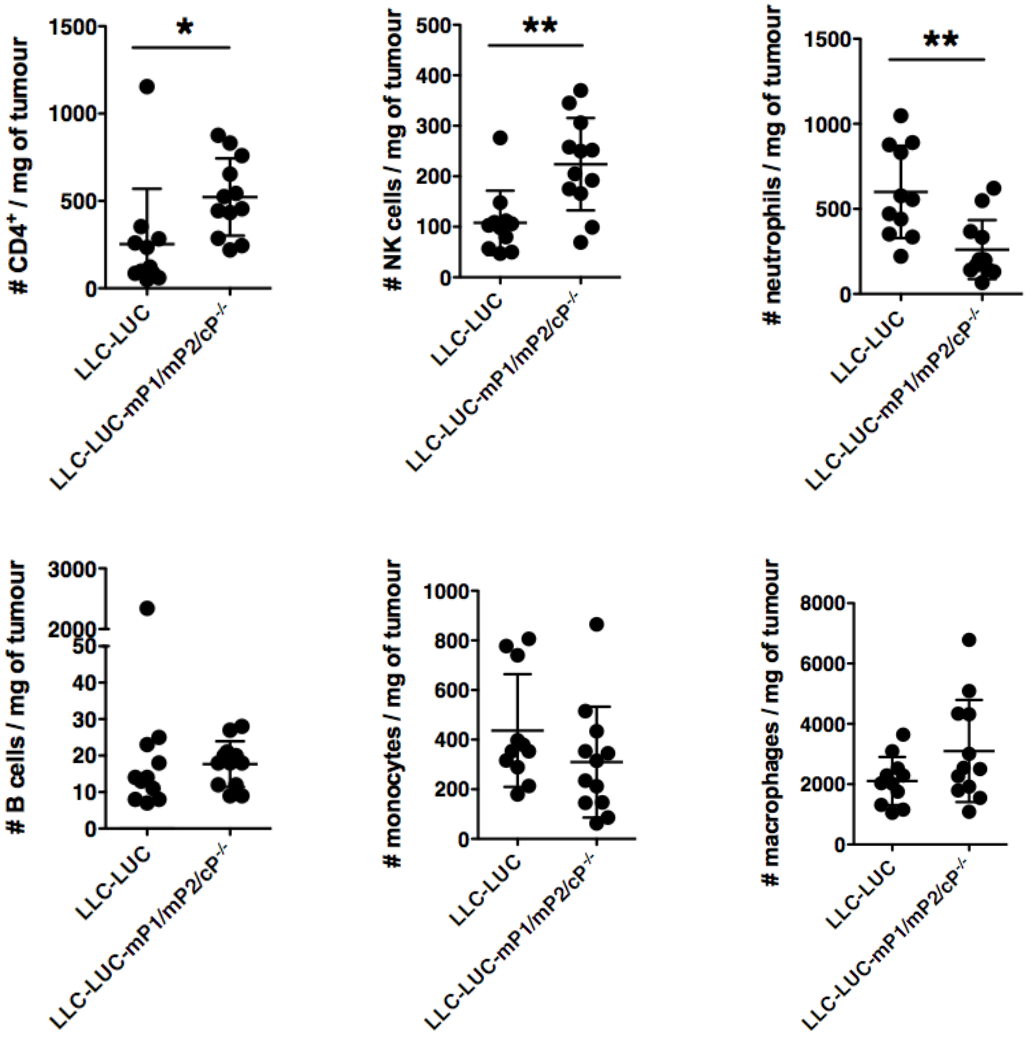


Figure 2. Absent PGE_2 production does not interfere with the growth of primary tumour, but changes in the immune infiltrate (**A**) Growth of primary tumours. (**B**) Immune infiltrate of primary tumours on the day of surgery (day 26). $CD4^+$ T cells (live $CD45.2^+$ $CD3^+$ $CD4^+$), NK cells (live $CD45.2^+$ $CD3^-$ $NK1.1^+$) and neutrophils (live $CD45.2^+$ $CD11b^+$ $Ly6G^+$). Dead cells were excluded from the analysis. Mean ± SD, *p<0.05 **p<0.005 (two-tailed Student's t-test with Welch's correction).

There was no difference in the growth of primary tumours between the groups (Fig. 2 A). Tumours were resected on day 26 followed by analysis of immune infiltrate by flow cytometry. LLC-LUC-mP1/mP2/cP$^{-/-}$ tumours contain more NK cells and CD4^{+} T cells, whereas numbers of neutrophils are reduced. No difference was observed in numbers of monocytes, macrophages and B cells between the two cohorts (Fig. 2 B).

Mice bearing tumours deficient in PGE_2 production are less prone to develop metastatic disease

Three weeks after resection of the primary tumours we have screened mice for the presence of metastatic lesions. Whereas 78% of control animals (7/9) developed metastases, only 27% in the LLC-LUC-mP1/mP2/cP$^{-/-}$ group (3/11) had metastases (Fig. 3). This points to an important role of tumour-derived PGE_2 in the metastatic cascade (Fig. 3).

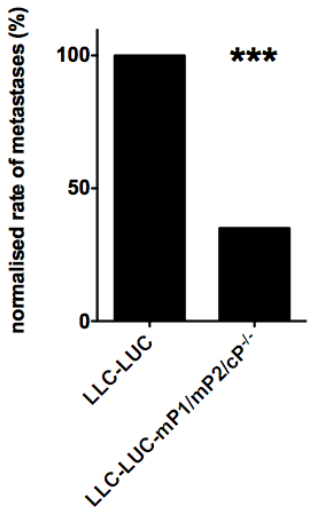


Figure 3. Production of PGE_2 is crucial for establishment of metastasis. Rate of metastasis normalized to the control group. ***p<0.0005 (Chi-square test).

3.2.4 Discussion

We show that PGE_2 derived from the tumour cells is sufficient for development of metastatic disease. As also macrophages and other myeloid cells can produce prostaglandins, it is likely that also this contributes to metastasis, but we did not formally address this here.

PGE_2 is a potent inducer of polarization of TAMs into protumorigenic M2 cells (*109*). As the majority of the leukocytes in the tumours are macrophages, it will be interesting to investigate

whether TAMs in the LLC-LUC-mP1/mP2/cP$^{-/-}$ tumours exhibit maturation differences as compared to those in the parental tumours. These could be measured by expression levels of arginase I or mannose receptor (CD206). Furthermore, protumorigenic macrophages produce high amounts of VEGFs (*161*). It is thus possible that LLC-LUC-mP1/mP2/cP$^{-/-}$ tumours contain higher amounts of VEGF and as a consequence have changes in lymph or blood vasculature, what could explain the decreased metastatic rate in these animals.

PGE_2 can also inhibit the function of $CD8^+$ T and NK cells (*60, 68*). The data described in chapter 3.1 show that NK cells are crucial to metastatic seeding. It is important to characterise both tumour and blood NK cells with respect to their maturation and expression of activating receptors. Furthermore, although there are no differences in numbers of $CD8^+$ T cells, we did not yet investigate their effector function.

Tumours can induce changes in distant organs, for example the accumulation of immature myeloid cells (*134, 136*), often referred to as the pre-metastatic niche. It is interesting to investigate whether tumour-derived PGE_2 impacts on the pre-metastatic niche during cancer progression. As we have observed decreased infiltration of neutrophils into the primary tumours, it is possible that mice have lower numbers of neutrophils in the circulation or potentially directly in the metastasis target organs, like lungs or livers. Neutrophils were previously shown to dampen $CD8^+$ T cell responses and inhibit NK cells from lysing circulating tumour cells (*124, 125*). Reduced neutrophil counts could also underline potential differences in activity of tumour-infiltrating lymphocytes.

It can be speculated that decrease in the metastatic burden is a result of insufficient autocrine signalling of prostaglandin, which could lead to increased apoptosis of metastasising tumour cells (*85*). This is however unlikely, as LLC-LUC-mP1/mP2/cP$^{-/-}$ tumours have similar growth kinetics *in vivo* to the parental cells.

Furthermore, when injected subcutaneously, tumours deficient in PGE_2 production grow equally fast as the parental LLC-LUC. This stands in contrast to the reported data showing that genetic deletion of COX-1 and COX-2 in melanoma cells induces immune-mediated rejection of tumours through both innate and adaptive immunity (*267*). This discrepancy may be explained by differences in immunogenicity or in tumour-associated immune infiltrate between melanoma cells and the LLC cells we used. It would be interesting to see if increasing the antigenicity of tumour cells, e.g. by inducing expression of an additional antigen would reveal an impact of PGE2 on tumour-specific T cells or on growth of the primary tumour. Alternatively, COX-1 and COX-2 are not specific for PGE_2 and can process PGH_2 into other prostanoids, like PGD_2, PGI_2, $PGF_{2\alpha}$, and thromboxane A_2 (TXA_2), which might partially explain the differences between studies (*268*).

It is also possible that primary LLC-LUC-mP1/mP2/cP$^{-/-}$ tumours grow equally well as the parental ones the loss of immunomodulatory function of PGE_2 is compensated by other

mechanisms. $CD4^+$ T cells better infiltrate lLC-LUC-mP1/mP2/$cP^{-/-}$ tumours and at this stage it cannot be excluded that these $CD4^+$ T cell are $FoxP3^+$ T regulatory cells.

The next step would be to define if LLC-LUC-mP1/mP2/$cP^{-/-}$ tumour cells can extravasate in the target organs as efficiently as the parental cell line. This can be addressed by intravenous injection of tumour cells. Depletion of NK cells in this context would also allow determining if LLC-LUC-mP1/mP2/$cP^{-/-}$ cells were susceptible to NK cell-mediated control.

Further experiments are required to delineate the mechanism by which tumour-derived PGE_2 promotes metastasis. Such data may ultimately open new perspectives for using anti-inflammatory drugs in prevention of metastatic disease.

3.3 The efficacy of agonistic anti-CD27 antibody, agonistic anti-CD40 and checkpoint blockade in protection against spontaneous metastases.

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3.3.1 Introduction

CD27 is a member of the TNF receptor superfamily expressed on T as well as NK cells (*282*). Its ligation stimulates $CD8^+$ T cell activation, survival and acquisition of memory phenotype, as well as IFN-γ production by CD4+ T cells (*283*). On the NK cells, CD27, in combination with CD11b, defines four stages of maturation, where immature NK cells are $CD27^-$ $CD11b^-$. Along maturation process, NK cells upregulate CD27 ($CD27^+$ $CD11b^+$), what is followed by increased CD11b expression ($CD27^+$ $CD11b^+$) and downregulation of CD27 in the final stage ($CD27^-$ $CD11b^+$) (*284*). Agonistic anti-CD27 antibodies have been tested in preclinical models of lymphoma and melanoma where they stimulate T as well as NK cell-mediated responses (*282, 283, 285*).

Also CD40 is also a member of the TNF receptor superfamily. It is expressed on APC, like B cells, DCs and monocytes (*38*). Ligation of CD40 by CD40L-expressing $CD4^+$ T helper cell drives APC maturation and licenses them for priming of $CD8^+$ T cells (*243*). This is achieved by increasing antigen presentation (signal 1), co-stimulation (signal 2) and secretion of cytokines, like type I interferons and IL-12 (signal 3) (*286*). Using a model of spontaneous metastasis we have shown that anti-CD40-mediated protection from metastases is driven mainly by NK cells (chapter 3.1).

Although anti-CD40 protects the majority of mice, about 10 - 20% still develop metastasis, suggesting that the anti-metastatic efficacy of anti-CD40 can be improved. We therefore supplemented anti-CD40 therapy with immune checkpoint blocking antibodies (anti-CTLA-4 + anti-PD-1). In addition, we investigated the protective potential of agonistic anti-CD27 antibody as a monotherapy.

3.3.2 Materials and methods

Mice

C57BL/6JOlaHsd1 mice were obtained from Harlan Laboratories (Envigo). Mice were kept under specific pathogen-free conditions at the Laboratory Animal Services Center at the University of Zurich.

Female mice of 6-8 weeks were used for all experiments. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by The Cantonal Veterinary Office Zurich.

Model of spontaneous metastasis

Mice were injected subcutaneously (s.c.) with 2×10^5 LLC-LUC cells in 100 µl PBS. To exclude the possibility that tumour size impacts on the rate of metastasis, we have excluded mice with tumours < 200 mg. Resection of primary tumours as well as detection of metastatic lesions was performed as described under 3.2.2.

Treatment of animals

Mice were injected with 100 µg anti-CD27 (clone RM27-3E5), 50 µg anti-CD40 (FGK45), 200 µg anti-CTLA-4 (9H10), 200 µg anti-PD-1 (RMP1-14) or 50 µg isotype antibody (2A3) per dose. Anti-CD40 and anti-CTLA-4 antibodies were purified from hybridoma culture supernatant using protein G sepharose 4 Fast Flow (Sigma-Aldrich). Anti-CD27 and anti-PD-1 were provided by Prof. Dr. Hideo Yagita. Isotype antibody was obtained from BioXCell. All antibodies were administered i.p. in 200 µl PBS.

Flow cytometry

Primary tumours were collected in PBS, cut into pieces and digested for 45 minutes at 37°C in RPMI medium containing 10% FBS, 1 mg/ml collagenase IV and 2.6 µg/ml DNase I (both Sigma-Aldrich). Samples were washed with PBS by centrifugation for 5 minutes at 350 *g*, the pellet was resuspended in PBS and filtered to remove debris. Blood samples were collected in PBS supplemented with 2% FCS (Invitrogen), 20 mM EDTA (Sigma Aldrich) and 0.03% NaN_3 (Sigma Aldrich). For surface staining antibodies against the following proteins were used: CD3 (17A2), CD4 (RM4-5), CD8α (53-6.7), CD11b (M1/70), CD19 (6D5), CD27 (LG.3A10), CD45.2 (104), F4/80 (BM8), KLRG1 (26), Ly6C (HK1.4), Ly6G (1A8), NK1.1 (PK136). For viability staining, Zombie Violet Fixable Viability Kit was used. Antibodies and viability stain were purchased from Biolegend. For surface and viability staining, samples were incubated in PBS for 25 minutes at 4°C. Subsequently, cells were washed and fixed for 25 minutes with 4% paraformaldehyde in PBS. FoxP3 staining was performed according to the manufacturer's protocol (Affymetrix eBioscience). For quantitative analysis, CountBright absolute counting beads were used (Thermo Fisher Scientific). Samples were acquired using a CyAn ADP 9 flow cytometer (Beckman Coulter) and analyzed using FlowJo v9.8.5 software

(Tree Star).

Measurement of inflammatory cytokines

Serum was collected using vacutainer rapid serum tubes (BD) and inflammatory cytokines were quantified using a CBA Mouse Inflammation Kit (BD).

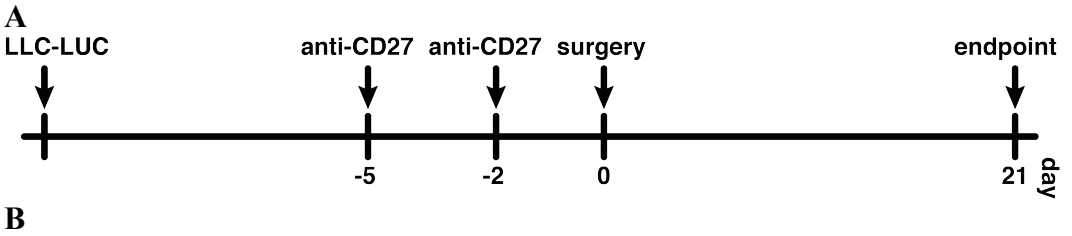
Statistical analysis

For comparison of two experimental groups, two-tailed Student's t-test with Welch's correction was performed. Rates of metastases were compared using chi-square test. All tests were performed with GraphPad Prism 5.0 and GraphPad QuickCalcs (GraphPad Software). *p<0.05, **p<0.005, ***p<0.0005.

3.3.3 Results

Anti-CD27 drives accumulation of maturate NK cells

Mice were injected s.c. with LLC-LUC tumours and treated with the first dose of anti-CD27 antibody when tumours were established (average 90 mm^2) (Fig. 1 A). In order to determine if anti-CD27 induced changes in peripheral blood leukocyte composition we took blood samples 24 hours after the first dose of anti-CD27. There were no differences in percentage of $CD8^+$, $CD4^+$ T nor NK cells (Fig. 1 B). However, NK cells in anti-CD27-treated group were more mature, as depicted by CD11b expression (Fig. 1 C). Based on these results, we measured the expression of killer cell lectin-like receptor subfamily G member 1 (KLRG1), a maturation and effector function marker, as well as stress receptor natural-killer group 2, member D (NKG2D). On the day of surgery, peripheral blood NK cells express higher levels of KLRG1, but not NKG2D (Fig. 1 D).



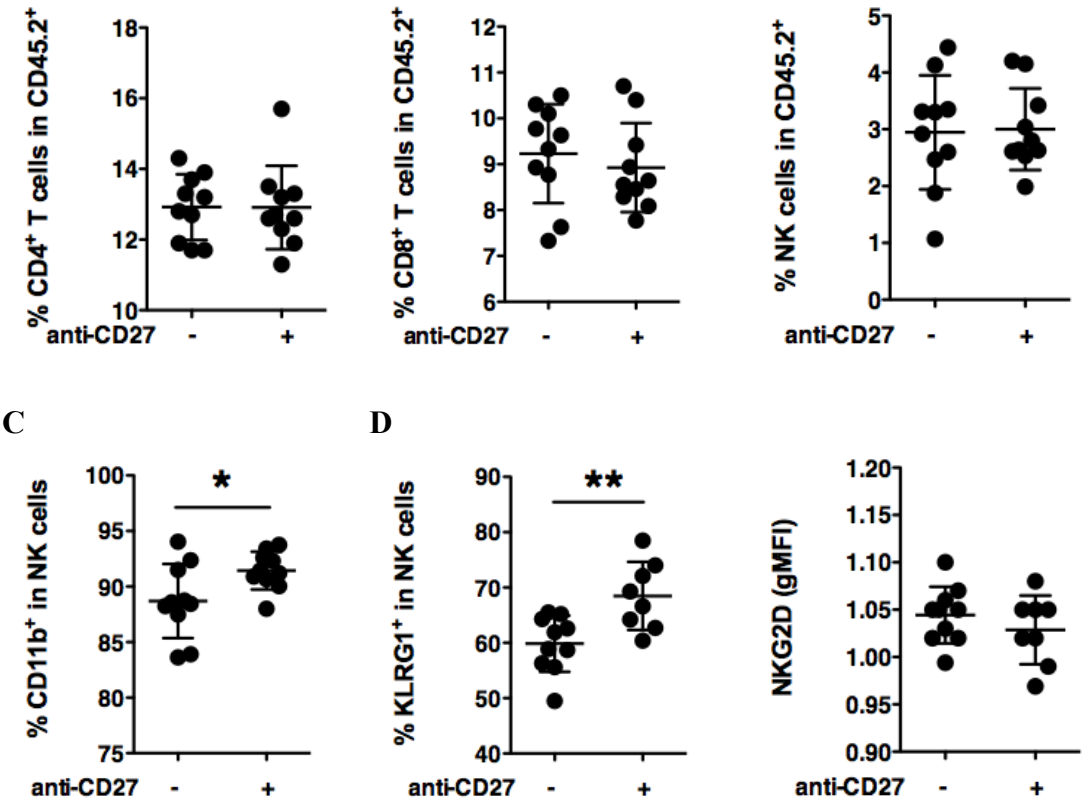


Figure 1. Administration of anti-CD27 does not change blood leukocyte composition, but drives maturation of NK cells. (**A**) Experimental timeline for anti-CD27 administration before the surgery. Primary tumours were resected on day 23 from the injection of tumour cells. (**B**) Percentage of $CD4^+$ ($CD45.2^+$ $CD3^+$ $CD4^+$), $CD8^+$ T ($CD45.2^+$ $CD3^+$ $CD8^+$), cells and NK ($CD45.2^+$ $CD3^-$ $NK1.1^+$), cells in total peripheral blood leukocytes on day -4. (**C**) Maturation of peripheral blood NK cells measured by CD11b expression on the day of surgery. (**D**) KLRG1 and NKG2D expression by peripheral blood NK cells. Blood samples taken on the day of surgery. Mean ± SD, $*p<0.05$ $**p<0.005$ (two-tailed Student's t-test with Welch's correction)

Furthermore, we have analysed the immune infiltrate of primary tumours. There were no differences in numbers of B, T and NK cells nor myeloid cells, including $Ly6C^+$ monocytes, macrophages and neutrophils (Fig. 2 A). As in the peripheral blood, tumour-infiltrating NK cells exhibited more mature, $KLRG1^+$ phenotype (Fig. 2 B). Agonistic anti-CD27 antibody had no impact on the size of primary tumour (Fig. 2 C).

A

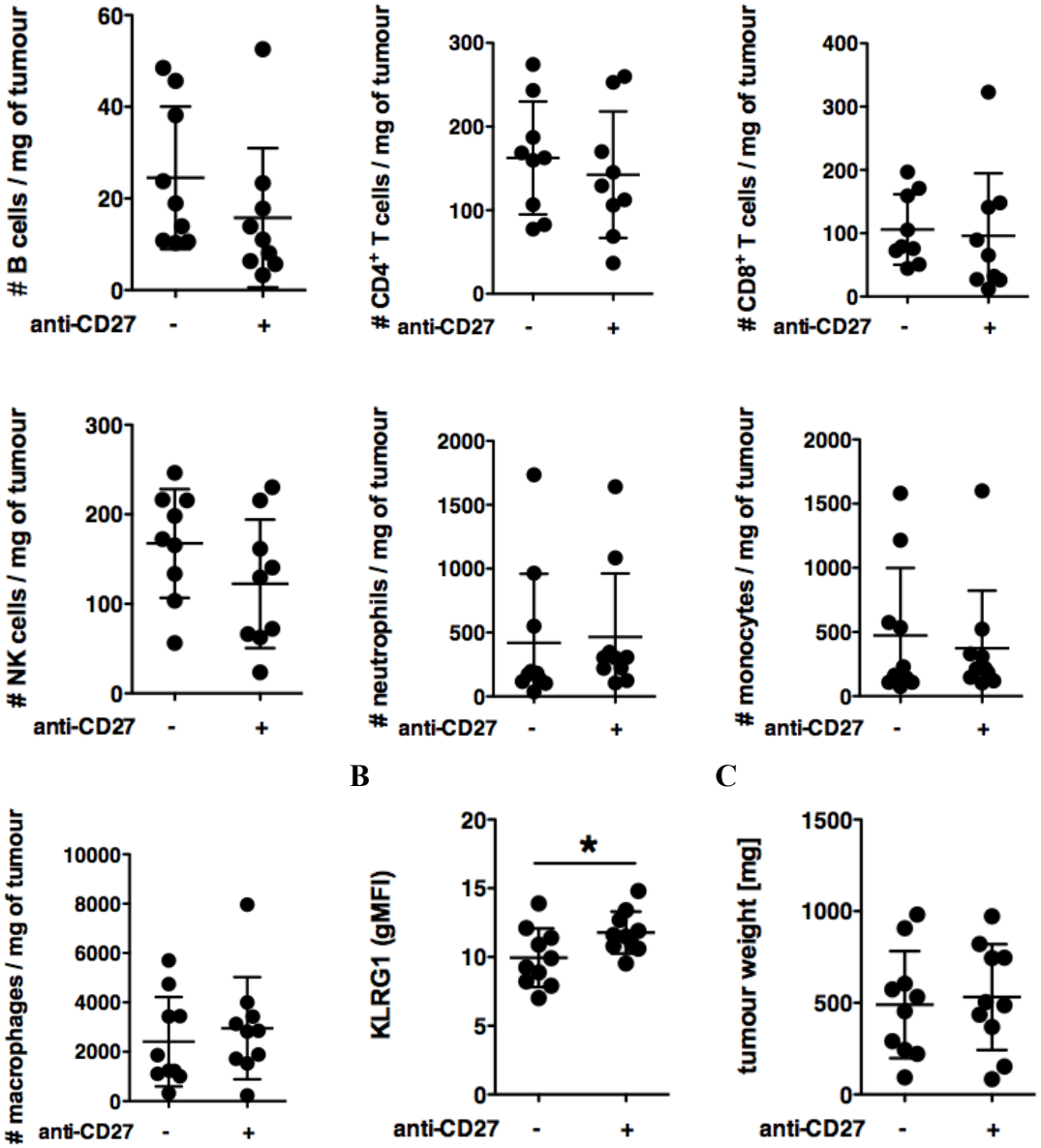


Figure 2. Changes induced by anti-CD27 in primary tumours resemble those from periphery. (**A**) Numbers of B (live CD45.2$^+$ CD19$^+$), CD4$^+$ (live CD45.2$^+$ CD3$^+$ CD4$^+$), and CD8$^+$ T cells (live CD45.2$^+$ CD3$^+$ CD8$^+$), NK cells (live CD45.2$^+$ CD3$^-$ NK1.1$^+$), neutrophils (live CD45.2$^+$ CD11b$^+$ Ly6G$^+$), inflammatory monocytes (live CD45.2$^+$ CD11b$^+$ Ly6C$^+$), and macrophages (live CD45.2$^+$ CD11b$^+$ F4/80$^+$ Ly6C^{int}), in the tumour on the day of surgery. (**B**) Expression of KLRG1 by tumour-infiltrating NK cells. (**C**) Weight of primary tumours. Mean ± SD, *p<0.05 (two-tailed Student's t-test with Welch's correction)

We therefore conclude that a single dose of anti-CD27 in tumour-bearing animals does not stimulate significant proliferation of NK or T cells, although it drives accumulation of mature NK cells.

Anti-CD27 does not stimulate systemic inflammation

In order to define if anti-CD27 induces a systemic inflammatory response, we have measured

serum levels of IL-6, IL-12, TNF, CCL-2 and IFN-γ as well as anti-inflammatory cytokine IL-10 24 h after the first dose of anti-CD27. We observed no induction of IL-6, TNF or CCL-2 (Fig. 3), whereas the amount of IL-10, IL-12 and IFN-γ were below the detection limit (data not shown).

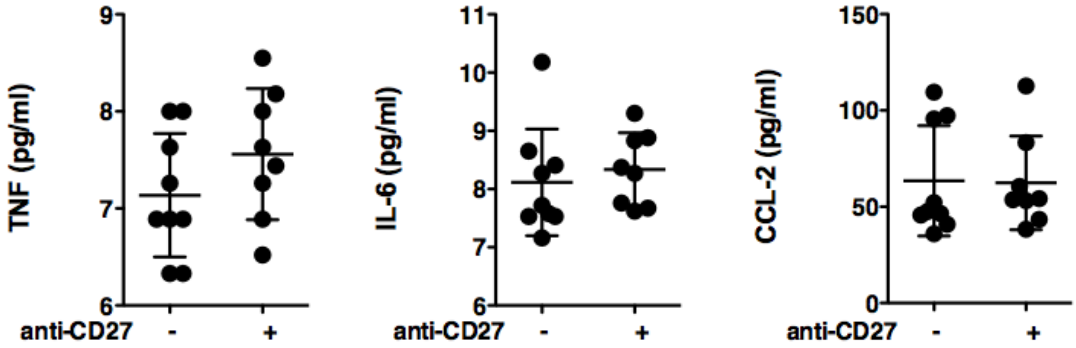


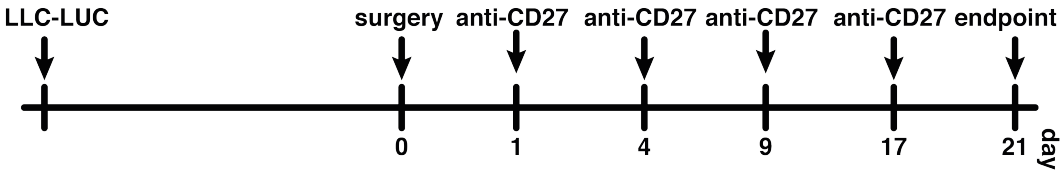
Figure 3. Single dose of anti-CD27 does not induce systemic inflammation. Amount of inflammatory cytokines in the serum 24 hours after the first injection of anti-CD27 (day -4). Mean ± SD.

Stimulation of immune system with anti-CD27 is not sufficient to treat metastatic disease

To determine if anti-CD27 impacts on the development of progression of metastatis, we have compared therapy started before the resection of primary tumour (Fig. 1 A) and after the surgery (Fig. 4 A). None of the regimens changed the rate of metastasis (Fig. 4 B).

Based on these results we conclude that agonistic anti-CD27 antibody as a monotherapy is not sufficient for inducing anti-metastatic effects in LLC-LUC tumour bearing animals.

A



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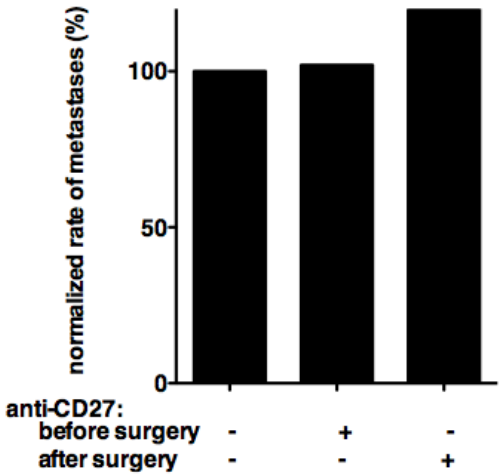

Figure 4. Anti-CD27 has no anti-metastatic effect when given neither before nor after the surgery. (**A**) Experimental timeline for anti-CD27 administration after the surgery. Primary tumours were resected on day 23 from the injection of tumour cells. (**B**) Rate of metastasis normalized to the control group.

Blockade of CTLA-4 and PD does not improve the anti-metastatic effect of agonistic anti-CD40 antibody

Because anti-CD40 has a significant anti-metastatic effect but did not prevent metastasis in all the mice (chapter 3.1), we have investigated whether additional stimulation of immunity with checkpoint blocking antibodies improves the anti-metastatic efficacy of anti-CD40 treatment. Anti-CD40 prevents development of metastatic disease only if given before the surgery. However, to sustain the elicited immune response we have continued checkpoint blockade treatment until the endpoint, according to the treatment scheme (Fig. 5 A). Tumours were resected on day 28 from tumour cell injection and we have analysed their immune infiltrate.

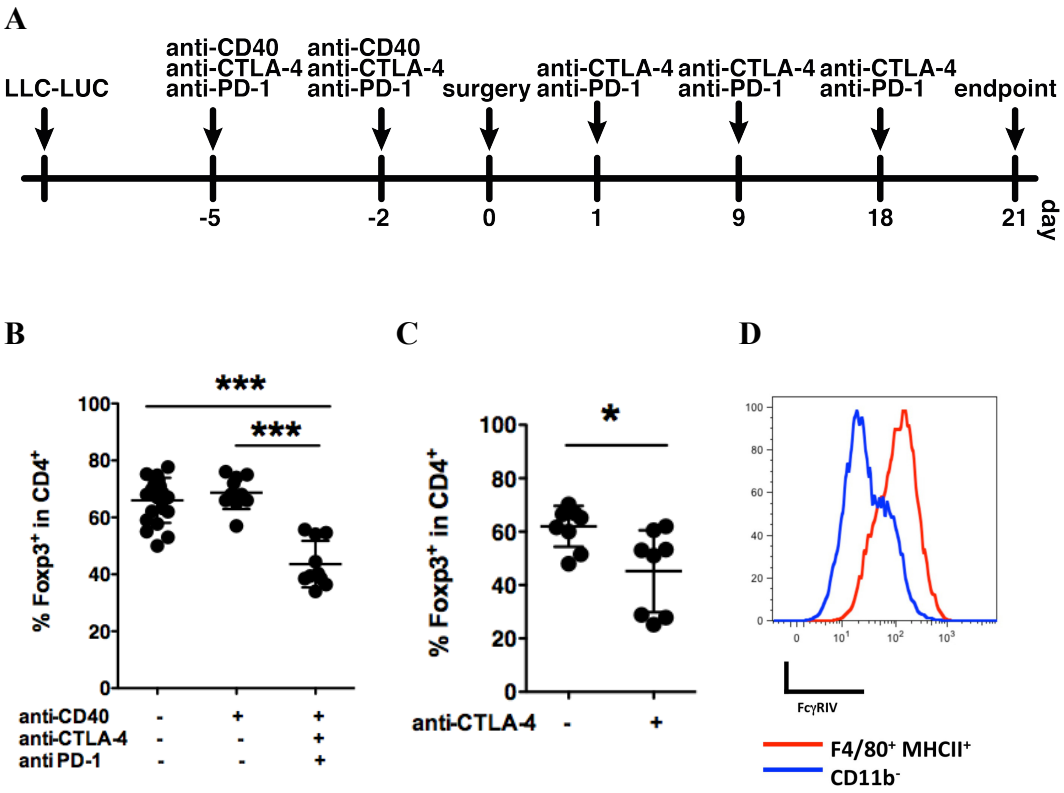

Figure 5. Combination of anti-CD40, anti-CTLA-4 and anti-PD-1 reduces percentage of Tregs in CD4$^+$ T cell pool. (**A**) Experimental time line of combination treatment starting before the surgery. Primary tumours were resected on day 28 from the injection of tumour cells. (**B**) Percentage of Tregs in total CD4$^+$ T cell infiltrate of primary tumour on the day of surgery. Mean ± SD, ***p<0.0005 (non-parametric Kruskal-Wallis tests with Dunn's post-tests). (**C**) Percentage of Tregs in total CD4$^+$ T cell (live CD45.2$^+$ CD3$^+$ CD4$^+$ FoxP3$^+$) infiltrate of primary tumour on the day of surgery. Mice were treated with anti-CTLA-4 on day -5 and -2. Mean ± SD, *p<0.05 (two-tailed Student's t-test with Welch's correction). (**D**) Expression of FcγRIV on MHCII$^+$ population of tumour-associated macrophages (live CD45.2$^+$ CD11b$^+$ F4/80$^+$ MHCII$^+$) as measured on the day of surgery.

Compared to the animals treated with anti-CD40 alone, we observed a reduced percentage of Tregs within CD4$^+$ T cell pool (Fig. 5 B). This is mediated by anti-CTLA-4 (Fig. 5 C) and is consistent with the observation that those anti-CTLA4 clones, which bind Fcγ-receptors, elicit antibody-dependent cell-mediated cytotoxicity (ADCC) resulting in depletion of CTLA-4^{high} Tregs (*66*). The anti-CTLA-4 clone 9H10 clone we have used binds FcγRIV (*66*), which is expressed on ADCC-competent macrophages in the infiltrate of LLC-LUC tumours (Fig. 5 D).

We have compared the anti-metastatic activity of combination of antibodies to anti-CD40 alone in the two regimens: starting before (Fig. 5 A, Fig. 6 A) and after the surgery (Fig. 6 B, C). In both regimens, combination of antibodies was not more beneficial than the anti-CD40 alone (Fig. 6 D, E). We conclude that in our model the anti-metastatic effect of anti-CD40 antibody cannot be improved by checkpoint blockade.

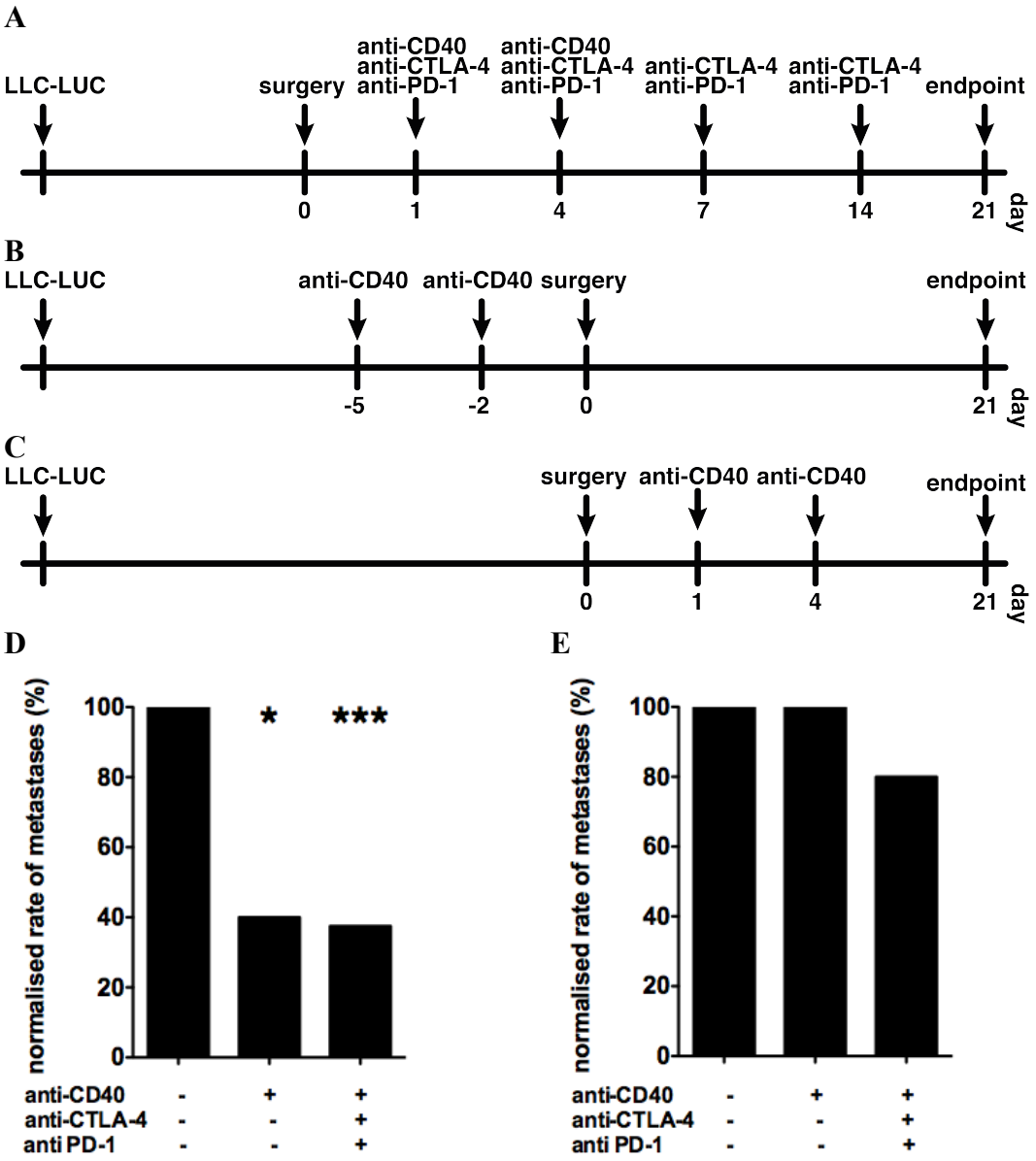

Figure 6. Supplementation of anti-CD40 therapy with anti-CTLA-4 and anti-PD-1 does not improve the anti-metastatic effect. (**A**) Experimental time line of combination treatment starting after the surgery. Primary tumours were resected on day 23 from the injection of tumour cells. (**B**) Experimental time line of anti-CD40 treatment starting before the surgery. Primary tumours were resected on day 23 from the injection of tumour cells. (**C**) Experimental time line of anti-CD40 treatment starting after the surgery. Primary tumours were resected on day 23 from the injection of tumour cells. (**D, E**) Rate of metastasis normalized to the control group. *p<0.05, ***p<0.0005 (Chi-square test)

3.3.4 Discussion

It was shown previously that agonistic anti-CD27 antibody reduced the number of metastasis after i.v. injection of B16-OVA cells in an IFN-γ dependent fashion (*283*). In contrast, we did

not observe an impact of anti-CD27 treatment on the growth of primary tumours or metastasis. As anti-CD27 promotes development of effector T cells, this discrepancy might be explained by the fact that B16-OVA cells are more immunogenic than the LLC-LUC cells we used. It is interesting to test this hypothesis by using LLC-LUC-OVA cells. However, another study that used a different clone of anti-CD27 also reported control of parental B16 melanoma cells (*283*). In both cases, mice were treated with seven injections of anti-CD27, whereas we have used only two. A two-dose regimen was used in another study to induce rejection of lymphomas, what was mediated by $CD8^+$ T cells with minor contribution of NK cells (*285*). In this reported experiment treatment was initiated on day six, when tumour were below 20 mm^2 (*285*), whereas we have started injecting anti-CD27 on day 18, when tumours reached size of approximately 60 mm^2. This means longer exposition of lymphocytes to immune-subversive tumour environment. It is possible that continued anti-CD27 treatment is necessary to sustain anti-tumour response to established tumour lesions and it could explain why we have not seen any impact on the size of primary tumours. It cannot however explain equal rates of metastasis between the treatment groups.

Besides stimulating T cell responses, agonistic anti-CD27 induces NK cell activation (*282, 285*). We have observed increased percentage of mature $CD11b^+$ and $KLRG1^+$ NK cells, but no differences in total NK cell numbers, arguing against major expansion of NK cells. The impact of anti-CD27 on NK cell cytotoxicity against LLC-LUC tumours needs to be further elucidated. Furthermore, we have shown previously that the anti-metastatic effect of anti-CD40 is dependent on NK cells without contribution of $CD8^+$ T cells (chapter 3.1). Analogously, it cannot be excluded that the anti-CD27 does not provide sufficient activation of NK cells, whereas in our model $CD8^+$ T cells are not able to control metastatic tumours.

In order to improve the anti-metastatic effect of anti-CD40 we have combined it with blocking anti-CTLA-4 and anti-PD-1 antibodies. Such therapy has the potential to mount an efficient T cell-mediated anti-tumour response (*223-226*) and, as NK cells also express PD-1 and CTLA-4, can improve the NK cell-mediated control of disseminated tumour cells (*208, 228, 229*). We have not observed therapeutic benefit of combination therapy compared to anti-CD40 alone, suggesting that in this experimental setup further activation of NK cells is not possible using checkpoint blocking antibodies. In addition, such therapy is insufficient to trigger T cell-mediated anti-metastatic response or shows that T cells play a minor role in controlling metastatic seeding. The latter is in agreement with our observation that CD8-depletion does not increase the metastatic load in our model (chapter 3.1).

The most prominent change triggered by the combination treatment in the immune infiltrate of primary tumours was reduced percentage of Tregs in the $CD4^+$ T cell pool. This is consistent with previous report (*66*) and can have potential effect on NK and $CD8^+$ T cell activity. On the other hand its impact may be overridden by deletion of total $CD4^+$ T cells that

is triggered by anti-CD40 alone (chapter 3.1). Consistently, we have not detected changes in the primary tumour size induced by the combination therapy, although it cannot be excluded that this could occur if treatment was initiated earlier, e.g. during the first week from injection of tumour cells.

Finally, none of the tested therapies were successful when treatment was started after the surgery, showing that although immune system can control the metastatic cascade on multiple steps, further work is required to establish a therapy that prevents both the establishment and outgrowth of the metastatic lesions.

3.4 Role of the activated leukocyte cell adhesion molecule (ALCAM) in the metastatic cascade

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* These results will be part of a manuscript by Willrodt, *et al*. (in preparation).

3.4.1 Introduction

Activated leukocyte cell adhesion molecule (ALCAM, CD166) is a member of the immunoglobulin superfamily and is expressed during steady state by a wide array of cells, including leukocytes, fibroblasts, epithelial cells, hepatocytes, neurons and mesenchymal as well as hematopoietic stem cells (*287, 288*). In addition, ALCAM expression is induced on lymphatic endothelium in the context of inflammation (*289*). ALCAM enables adhesion through binding with CD6, L1CAM, galectin-8 or homophilic interaction with other ALCAM molecules (*287, 289*).

ALCAM participates in leukocyte transmigration through endothelial walls, but is also involved in angiogenesis and in interaction between DCs and lymphocytes (*287, 288*). Furthermore, blockade of ALCAM reduces DC adhesion to lymphatic endothelial cells (*289*).

Cancer cells can express ALCAM, presumably as a result of inflammatory signalling and NFκB activation (*290*). More than 70% of melanomas are ALCAM-positive and it is suggested that it contributes to metastasis by enabling adhesion of circulating tumour cells to the endothelium (*290*). Furthermore, ALCAM is a marker of colorectal cancer stem cells (*288*). Overexpression of soluble ALCAM by cancer cells, which blocks ALCAM interactions, reduces the amount of lung metastases in nude mice (*290*). Alternatively, ALCAM may induce a change in the cellular pathways linking proliferation and invasiveness, as was shown for other adhesion molecules like E-cadherin and L1CAM (*287*).

Here we show that *Alcam*-deficient mice develop significantly less metastasis from resected LLC-LUC tumours compared to wild type mice.

3.4.2 Materials and methods

Mice

C57BL/6J mice were obtained from Jackson Laboratory. B6.129(FVB)-*Alcam*^{tm1Jawe}/J

(*Alcam*$^{-/-}$) mice were originally obtained from Jackson Laboratory and bred under specific pathogen-free conditions at the Rodent Center HCI ETH Zurich.

During experiments mice were kept under specific pathogen-free conditions at the Laboratory Animal Services Center at the University of Zurich. Female mice of 6-8 weeks were used for all experiments. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by The Cantonal Veterinary Office Zurich.

Model of spontaneous metastasis

Mice were injected subcutaneously (s.c.) with 2×10^5 LLC-LUC cells in 100 µl PBS. To exclude the possibility that tumour size impacts on the rate of metastasis, we have excluded mice with tumours below 200 mg and over 1000 mg. Resection of primary tumours as well as detection of metastatic lesions was performed as described under 3.2.2.

Flow cytometry

Blood samples were collected in PBS supplemented with 2% FCS (Invitrogen), 20 mM EDTA (Sigma Aldrich), 0.03% NaN_3 (Sigma Aldrich). Primary tumours were collected in PBS, cut into pieces and digested for 45 minutes at 37°C in RPMI medium containing 10% FBS, 1 mg/ml collagenase IV and 2.6 µg/ml DNase I (both Sigma-Aldrich). Samples were washed with PBS by centrifugation for 5 minutes at 350 *g*, the pellet was resuspended in PBS and filtered to remove debris. For surface staining antibodies against the following proteins were used: CD3 (17A2), CD4 (RM4-5), CD11b (M1/70), CD19 (6D5), CD45.2 (104), F4/80 (BM8), Ly6C (HK1.4), Ly6G (1A8), NK1.1 (PK136). For viability staining, Zombie Violet Fixable Viability Kit was used. Antibodies and viability stain were purchased from Biolegend. For surface and viability staining, samples were incubated in PBS for 25 minutes at 4°C. For quantitative analysis, CountBright absolute counting beads were used (Thermo Fisher Scientific). Samples were acquired using a CyAn ADP 9 flow cytometer (Beckman Coulter) and analyzed using FlowJo v9.8.5 software (Tree Star).

Statistical analysis

For comparison of two experimental groups, two-tailed Student's t-test with Welch's correction was performed. Rates of metastases were compared using chi-square test. All tests were performed with GraphPad Prism 5.0 and GraphPad QuickCalcs (GraphPad Software). *p<0.05, **p<0.005, ***p<0.0005.

3.4.3 Results

Naïve and tumour-bearing Alcam-deficient mice show changes in circulating leukocyte

During the metastatic cascade immune cells can interact with tumour cells in three environments: in the primary tumour, circulation and the target organ. We have thus analysed leukocyte populations in these three compartments.

In tumour-naïve *Alcam*$^{-/-}$ mice we observed elevated leukocytes counts in the peripheral blood (Fig.1 A). This can be partially explained by increased numbers of granulocytes and monocytes (Fig. 1 B, C). Among the total monocyte population in the blood of *Alcam*$^{-/-}$ mice there is lower percentage of inflammatory, Ly6C^{+} monocytes (Fig. 1 G) Numbers of NK cells (Fig. 1 D) were not changed although NK cells express a lower amount of the maturation marker CD11b (Fig. 1H). The numbers of circulating CD4^{+} and CD8^{+} T cells were reduced (Fig. 1 E, F). Leukocyte numbers in lungs and liver were similar to those in wild type mice (data not shown).

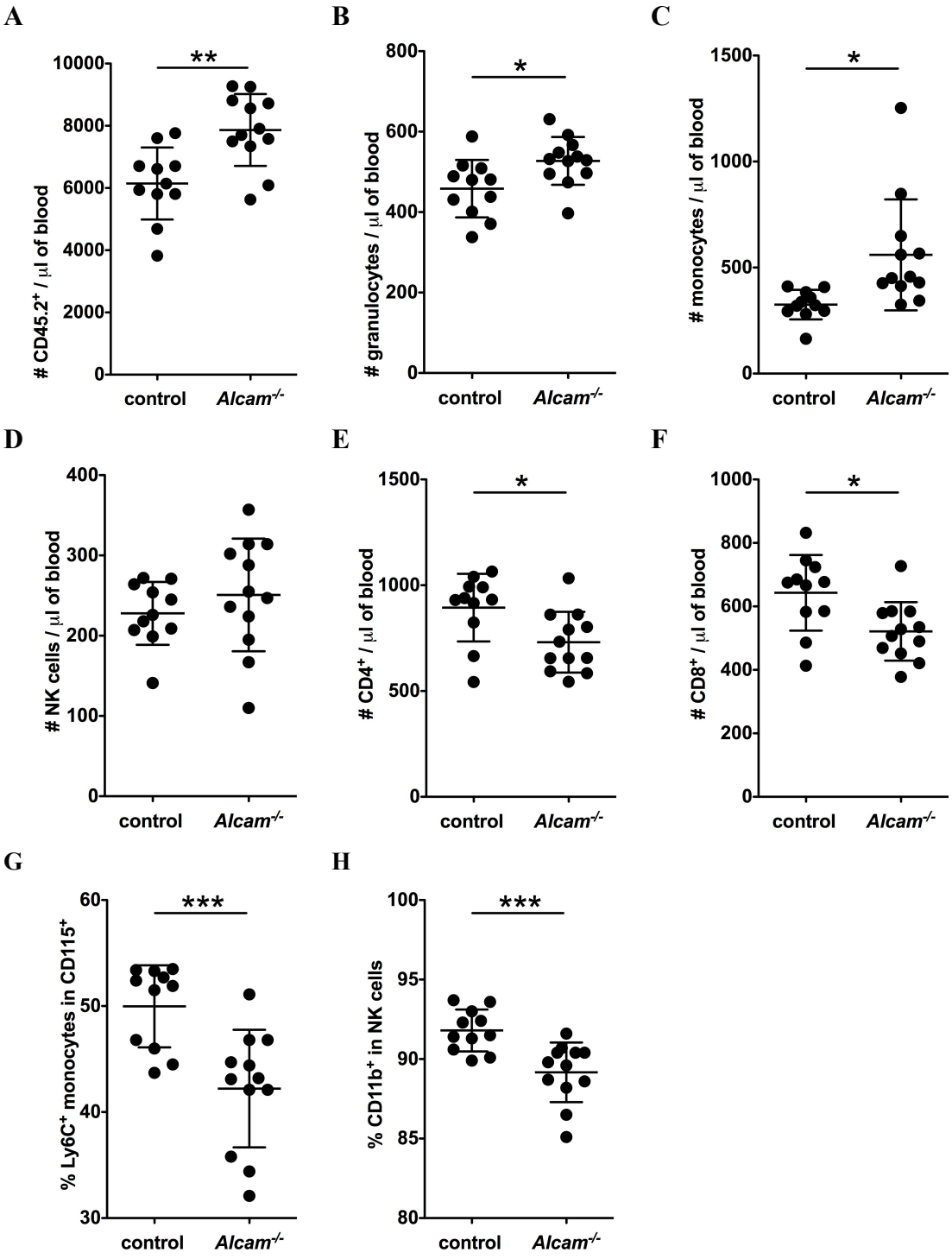


Figure 1. Naïve *Alcam*$^{-/-}$ mice exhibit changes in the peripheral blood leukocytes as compared to the control mice. (**A**) Total leukocyte counts in peripheral blood of *Alcam*$^{-/-}$ mice. Blood counts of granulocytes (CD45.2^{+} CD11b^{+} SS^{high}) (**B**), monocytes (CD45.2^{+} CD11b^{+} CSF1R^{+}) (**C**), NK cells (CD45.2^{+} CD3^{-} NK1.1^{+}) (**D**), CD4^{+} (CD45.2^{+} CD3^{+} CD4^{+}) (**E**) and CD8^{+} T cells (CD45.2^{+} CD3^{+} CD8^{+}) (**F**). (**G**) Percentage of inflammatory, Ly6C^{hi} monocytes in the total monocyte pool. (**H**) Percentage of CD11b^{+} NK cells in the total NK cell pool. Mean ± SD, *p<0.05 **p<0.005 ***p<0.0005 (two-tailed Student's t-test with Welch's correction).

To determine if the changes in blood leukocyte counts are sustained in the context of tumour development, we have injected mice with LLC-LUC tumour cells and analysed blood on day 14, what corresponds to tumour size of approximately 60 mm^2. As in tumour-naïve mice, total leukocyte counts were elevated (Fig. 2 B), as were granulocytes (Fig. 2 C) and monocytes (Fig. 2 D). NK cells were constantly expressing lower levels of CD11b, suggesting tumour-independent changes in NK cell maturation (Fig. 2 H). There was however no difference in numbers of T cells (Fig. 2 E, F) nor in the percentage of inflammatory monocytes (Fig. 2 G).

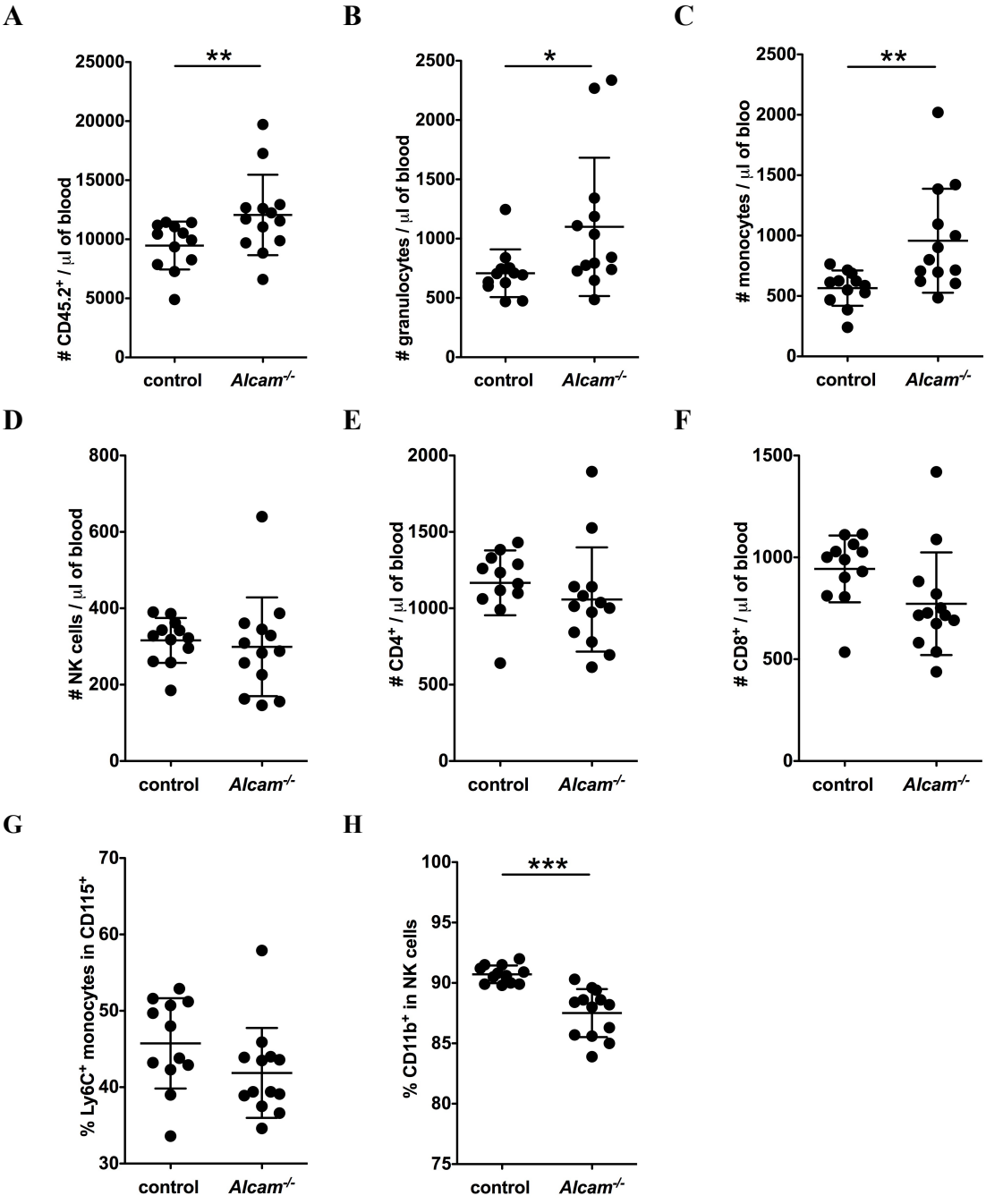
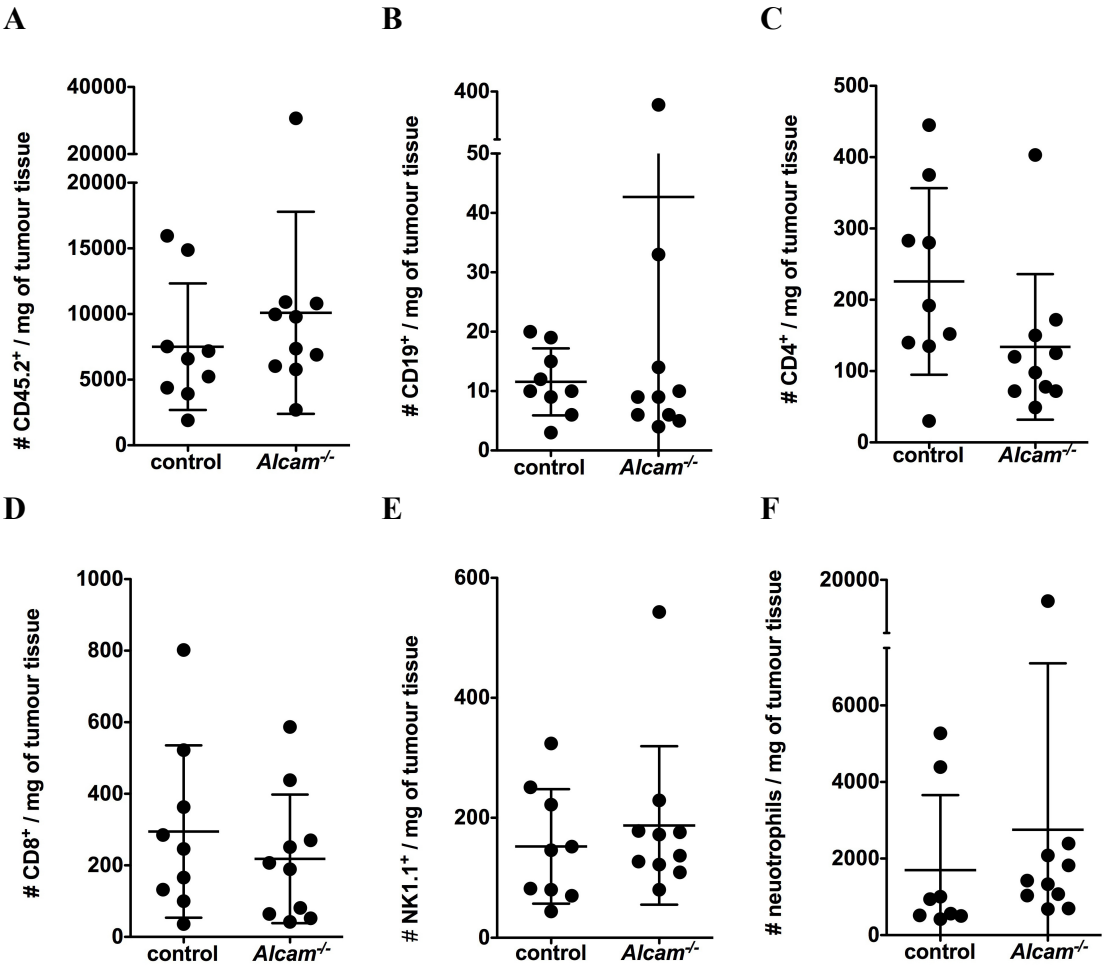


Figure 2. Changes in blood leukocyte counts *Alcam*$^{-/-}$ are partially sustained in tumour-

bearing animals. (**A**) Total leukocyte counts in peripheral blood of *ALCAM*$^{-/-}$ mice. Blood counts of granulocytes (CD45.2^{+} CD11b^{+} SS^{high}) (**B**), monocytes (CD45.2^{+} CD11b^{+} CSF1R^{+}) (**C**), NK cells (CD45.2^{+} CD3^{-} NK1.1^{+}) (**D**), CD4^{+} (CD45.2^{+} CD3^{+} CD4^{+}) (**E**) and CD8^{+} T cells (CD45.2^{+} CD3^{+} CD8^{+}) (**F**). (**G**) Percentage of inflammatory, Ly6C^{hi} monocytes in the total monocyte pool. (**H**) Percentage of CD11b^{+} NK cells in the total NK cell pool. Mean ± SD, *p<0.05 **p<0.005 ***p<0.0005 (two-tailed Student's t-test with Welch's correction).

Because circulating immune cells in *Alcam-/-* differ from those in wild type mice with respect to number and subset distribution, we analysed the leukocyte infiltrate in established LLC-LUC tumours (d 24). We observed no differences between control and *Alcam*$^{-/-}$ mice in any of the leukocyte populations analysed (Fig. 3



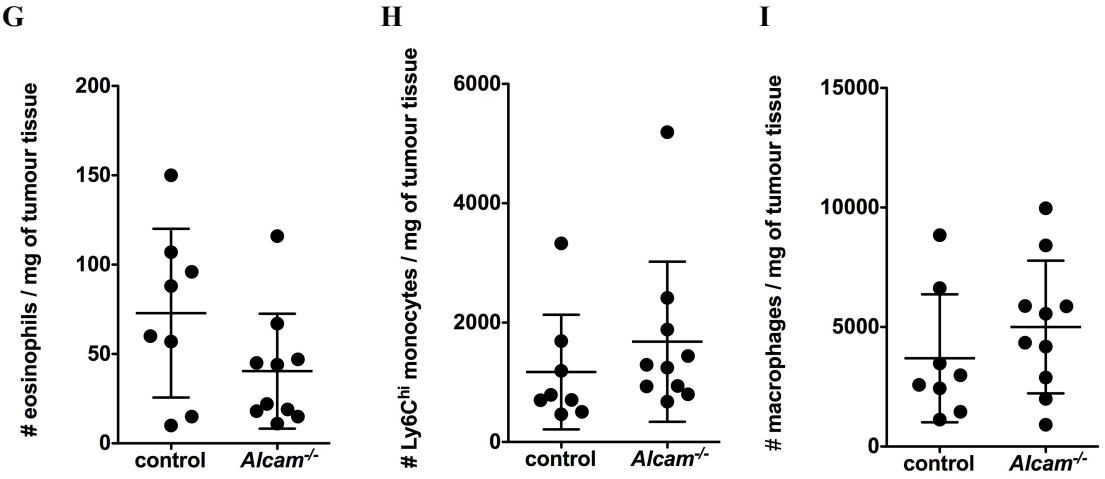


Figure 3. *Alcam*$^{-/-}$ and control mice have similar composition of the immune infiltrate of the primary tumour (**A**) Leukocyte counts in primary tumours of *Alcam*$^{-/-}$ mice. Numbers of B cells in the primary tumour infiltrate (live CD45.2^{+} CD19^{+}) (**B**), CD4^{+} (live CD45.2^{+} CD3^{+} CD4^{+}) (**C**) and CD8^{+} T cells (live CD45.2^{+} CD3^{+} CD8^{+}) (**D**), NK cells (live CD45.2^{+} CD3^{-} NK1.1^{+}) (**E**), neutrophils (live CD45.2^{+} CD11b^{+} Ly6G^{+}) (**F**), eosinophils (live CD45.2^{+} CD11b^{+} Siglec F^{+}) (**G**), Ly6C^{hi} monocytes (live CD45.2^{+} CD11b^{+} Ly6C^{hi}) (**H**) and macrophages (live CD45.2^{+} CD11b^{+} Ly6C^{int}) (**I**). Mean ± SD.

Alcam$^{-/-}$ *mice are protected against spontaneous metastasis*

Three weeks after resection of primary tumours, mice were sacrificed and analysed for the presence of metastatic lesions.

Out of nine mice in the control cohort, five developed metastasis (56%), whereas in the *Alcam*$^{-/-}$ group only two out nine (22%) (Fig. 4). We conclude that absence of ALCAM in the host partially protects from metastatic disease.

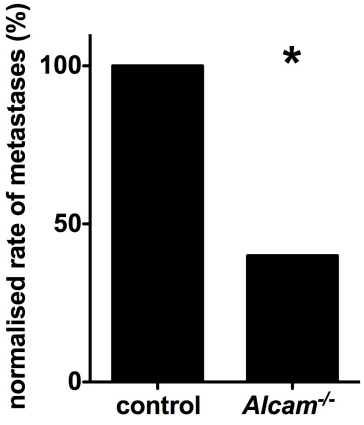


Figure 4. *Alcam*$^{-/-}$ mice are less prone to develop metastatic disease. Rate of metastasis

normalized to the control group. *p<0.05 (Chi-square test)

3.3.3 Discussion

We have observed that deletion of the *Alcam* gene in host reduces the metastatic potential of tumour cells. In order to establish a metastatic lesion, tumour cells have to intravasate into vessels of the primary tumour, survive in the circulation, extravasate through the endothelium and overcome potentially hostile environment of the target organ (*2*). Adhesion molecules, like ALCAM, influence this process in multiple stages.

First of all, it is possible that ALCAM interactions are important for tumour cell migration through endothelial barriers, as it is for leukocytes (*287, 288*). This is achieved by ALCAM-mediated adhesion to the vasculature and by affecting this step, tumour cells would not be able to form metastases because of inefficient seeding from the primary tumours. The same disrupted ALCAM-dependent adhesion to endothelium could block tumour cell extravasation in the target organ, thus protect from forming micrometastases in an immune system-independent manner.

Alternatively, the effect on metastasis might be caused by disrupted adhesion of leukocytes. The intra- and extravasation of tumour cells is facilitated by interaction with macrophages in the primary tumours as well as inflammatory monocytes in the metastatic organ. These cells induce vascular permeability by local production of VEGF, TGF-β and MMP-1 (*157, 158, 178*). In a similar manner, neutrophils activate endothelial cells through IL-1β and secrete matrix-modifying enzymes (*124*). They can also locally inhibit NK and $CD8^+$ T cells, thus protecting tumour cells during extravasation and establishment of micrometastases (*124, 125*). In the absence of interaction with myeloid cells, tumour cells might not be able to intravasate in the primary tumours nor extravasate and survive in the parenchyma of the target organ. Disturbed adhesion to endothelium could also explain elevated blood counts of monocytes and granulocytes in both naïve and tumour bearing mice.

Alternatively, the reduced metastatic rates could be explained by the beneficial effect of increased numbers of $Ly6C^{low}$ monocytes. This population of cells is responsible for patrolling of the blood endothelium, can sense invading tumour cells and attract cytotoxic cells, like NK cells (*149*). This would lead to increased lysis of metastatic tumour cells before they establish micrometastases.

Furthermore, disrupted migration of leukocytes could potentially affect the ability of immune cells to control extravasated tumour cells in the target organs.

Finally, it cannot be excluded that the difference we observe is directly linked with changes

NK cell activity. NK cells were shown to play a crucial role in control of metastatic tumour cells (*170*). *Alcam*$^{-/-}$ mice have elevated numbers of immature CD11b^{-} NK cells and this is independent of the presence of primary tumours. It is unclear weather murine NK cells cytotoxicity changes with maturation. It was reported however that compared to splenic cells, lung NK cells have lower cytotoxic activity, what was directly correlated with bigger fraction of fully mature, CD11b^{+} CD27^{-} NK cells in this organ (*291*). The same has been reported for the two populations of liver NK cells that differ regarding their CD11b expression (*147*). This opens the possibility that NK cells in *Alcam*$^{-/-}$ mice may be more proficient in lysis of metastatic tumour cells. Characterisation of NK cells in the *ALCAM*$^{-/-}$ animals would give evidence for this hypothesis.

LLC-LUC used in our model cells express ALCAM (data not shown). It remains to be seen if similar reduction in metastatic potential could be achieved by tumour-specific ablation of this adhesion molecule. This would point towards the homotypic ALCAM- ALCAM interactions as one of the crucial mechanisms underlying the metastatic cascade and explain the increased ALCAM expression on the metastatic cancer cells (*290*). However, at this stage, it cannot be excluded that the observed effect is due to tumour-intrinsic production of other ALCAM ligands, like CD6, galectin-8 or L1CAM (*287, 289*).

Our data show that expression of ALCAM on host cells is important for the development of metastatic disease. Further experiments are necessary to delineate the precise mechanism and define if this phenomenon depends on tumour cell interaction with immune cells or vasculature endothelium.

4. Discussion

Recent years have seen major progress in the development of immunotherapies. Antibodies, adoptive transfer of tumour-specific T cells, cytokines, TLR agonists and anti-cancer vaccines are becoming standard treatment for cancer patients. This is only possible because of the progress made in our understanding of the interaction between cancer and the immune system.

It is known since a long time that tumours are infiltrated by leukocytes (*292*), but only recently we have started understanding the complexity of the immune response in the tumour context. Different innate and adaptive immune cells are involved in each step of tumour progression and tumours actively sculpt their phenotype. Much of our understanding of these processes comes from studies focused on T lymphocytes and initially, therapies aimed to directly activate cytotoxic T cells. More recent data suggest that also innate immune cells including NK cells and ILCs are promising therapeutic targets.

The mutual interaction between cancer and the immune system has been integrated in the concept of immunoediting, in which tumours adapt to immunological pressure and as a result escape from immune control. Immunoediting assumes three phases of interaction between the immune system and cancer cells (*41*):

- Elimination, when tumours are killed by the immune system
- Equilibrium, in which tumour are kept in check by leukocytes, but are not eradicated
- Escape, when tumours progress due to acquisition of features that enable avoidance of immune surveillance.

This scheme is observed in both primary tumours as well as in metastatic lesions, suggesting that immunotherapy can treat metastases, even once the control of primary tumour has been lost.

Because insufficient activation of APCs is one of the major stumble stones for the induction of cancer-specific immune defence, we investigated whether APC activation by agonistic anti-CD40 antibody protects against metastasis. Anti-CD40 has been used to stimulate T cell-mediated immunity (*293-295*), but was shown also to activate NK cells (*296*), macrophages (*297*) and other cell types, including B cells (*298*). However, in majority of the studies the therapy was started when the tumour burden was low and none of them focused on the formation of metastasis. On the contrary, we have used anti-CD40 in advanced tumours and monitored reaction to both primary and metastatic lesions.

We observed that anti-CD40 prevents metastatic disease in an NK cell-dependent fashion. The anti-metastatic effect was only seen when anti-CD40 was administered before resection of the primary tumour. We interpret this finding such that anti-CD40 activates NK cells to

eliminate circulating tumour cells, of which the primary tumour is a continuous source.

In an attempt to potentiate anti-CD40 treatment, we used a small molecule inhibitor of CSF1R to deplete tumour-infiltrating macrophages. Unexpectedly, we observed that this nullifies the protective effect of anti-CD40. Systemic targeting of CSF1R-positive cells deregulates NK cell homeostasis, which has negative effect on the control of metastasising tumour cells. We have not observed beneficial effects of CSF1R inhibition on the primary tumour or on the outgrowth of metastatic lesions, although such observations were reported in other models (*106, 114-116, 254, 255*). However, none of these studies monitored development of metastatic disease.

Both anti-CD40 and CSF1R-targeting are currently tested in clinical trials (*38, 242, 256, 299*). Our results support the notion that NK cell activation can be used in therapy of metastatic tumours. This can be achieved by adoptive transfer approach, application of cytokines or, as we have shown here, activating leukocytes that orchestrate the immune response. Furthermore, our data suggest that loss of NK cells as a result of CSF1R inhibition can be prevented by supplementation of survival factors for NK cells, as shown here for IL-15.

We have tried to improve anti-CD40 therapy by combining it with two clinically relevant drugs – anti-CTLA-4 and anti-PD-1 antibodies. We have not observed improved control of metastasis compared to anti-CD40 alone, suggesting that checkpoint blockade does not have an additive or synergistic effect when given together with anti-CD40. Furthermore, the use of agonistic anti-CD27 antibody as monotherapy had not impact on development or progression of metastasis. These data suggest that therapies, which mainly impact on T cells, have limited capacity to prevent metastatic seeding or short-term outgrowth in the model we used here. This may be explained by our observation that T cells seem to play a minor role in these processes. T cells relatively poorly infiltrate LLC-LUC tumours and the control of metastasis seems to largely depend on NK cells. It remains to be clarified to what degree NK cells contribute to the therapeutic effect of checkpoint blockade and anti-CD27 therapy. In case of more immunogenic tumours, however, checkpoint blockade or anti-CD27 may have an anti-metastatic effect.

The importance of tumour-promoting inflammation is underlined by our findings regarding the prometastatic role of prostaglandins. Genetic engineering of LLC tumour cells to prevent the production of PGE_2 significantly reduced the capacity to metastasise, although it did not influence the kinetics of primary tumour growth. Our study shows that cancer-derived PGE_2 is sufficient to drive metastatic cascade, although the detailed mechanism needs to be further clarified. Most probable explanations include direct effect of PGE_2 on macrophage and neutrophil polarization, which contributes to the immunosuppressive tumour microenvironment (*60, 267-269*). Furthermore, PGE_2 alone is an angiogenesis-stimulating

factor (*161, 268*) and stimulates aggregation of platelets. The latter could prevent NK cell-mediated clearance of CTCs (*3, 172, 173, 300*).

In clinics, prostaglandin synthesis is targeted by blockade of cyclooxygenases with non-steroidal anti-inflammatory drugs (*268*). These can be used as adjuvants to conventional therapy (*273, 301*) and aspirin, a non-specific inhibitor of COX-1/COX-2 and platelet aggregation, has been linked to prevention of colon adenomas (*302*). This might open possibility to use anti-inflammatory therapeutics to block tumour-intrinsic chronic inflammation. Because acute inflammation is an essential stimulus for the initiation of immune responses, anti-inflammatory drugs should be applied with great care. Therefore, further understanding of cancer-associated inflammation is needed to block chronic inflammation and allowing protective immunity response at the same time.

Our observation that deletion of the adhesion molecule ALCAM in the host is sufficient to prevent metastasis but not the growth of primary tumours is interesting. At this point, however, we do not sufficiently understand which steps of the metastatic cascade are affected in *Alcam*$^{-/-}$ mice. The fact that many different cell types including leukocytes, cells of mesenchymal origin, endothelial and epithelial cells express ALCAM, makes it hard to predict which interactions are essential for metastasis. Using mice in which ALCAM is absent in distinct cell types may help to elucidate the role of ALCAM in metastasis.

In conclusion, successful cancer immunotherapy should trigger pathways of both innate and adaptive immunity and target primary lesions as well as disseminated tumour cells. This will be most probably achieved by combination of various classes of therapeutics. However, until we fully understand the mechanisms ruling immunity and its crosstalk with cancer cells, we have to be careful when designing novel drugs in order to avoid triggering detrimental side effects. Improving our understanding of tumour immunology paves the way for new therapies in the benefit of cancer patients.

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6. Appendix

6.1 Rational combination of immunotherapies with clinical efficacy in mice with advanced cancer

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Research Article

Cancer Immunology Research

Rational Combination of Immunotherapies with Clinical Efficacy in Mice with Advanced Cancer

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Abstract

In the context of cancer, naïve T cells are insufficiently primed and become progressively dysfunctional. Boosting antitumor responses by blocking PD-1 or CTLA-4 results in durable clinical responses only in a limited proportion of cancer patients, suggesting that other pathways must be targeted to improve clinical efficacy. Our preclinical study in TRAMP mice comparing 14 different immune interventions identified anti-CD40 + IL2/anti-IL2 complexes + IL12Fc as a uniquely efficacious treatment that prevents tolerance induction, promotes priming of sustained, protective tumor-specific $CD8^+$ T cells, and cures late-stage cancer when given together with adoptively transferred tumor-specific T cells. We propose that improving signals 2 (costimulation) and 3 (cytokines) together with fresh tumor-specific, rather than boosting of dysfunctional preexisting memory, T cells represents a potent therapy for advanced cancer. *Cancer Immunol Res; 3(11); 1279–88. ©2015 AACR.*

Introduction

The immune system plays a tumor-promoting and -suppressing role (1). Tumor-infiltrating Th1 and cytotoxic T cells are positive prognostic factors in human cancers (2); however, advanced tumors can evade immune attack and suppress tumor-specific immunity, which also limits the efficacy of standard therapies (1).

Immunotherapy aims to improve the protective effector function of tumor-specific T cells, which relies on 3 signals: stimulation of the T-cell receptor (TCR) receptor (signal 1), costimulation (signal 2), and cytokines (signal 3; ref. 3). Immature dendritic cells (DC) do not sufficiently provide signals 2 and 3 and induce robust, antigen-specific T-cell tolerance (4–6). Immunotherapies boosting tumor-specific immunity in mice and humans include vaccines (7), adoptive cell transfers (8), induction of antigen-presenting cell (APC) maturation (9, 10), checkpoint blockade (11, 12), and cytokines (13, 14), but the frequency of responders remains low.

We therefore aimed to prime tumor-specific $CD8^+$ T cells, prevent induction of tolerance, and achieve control of large, established tumors. We used TRAMP mice that develop autochthonous prostate cancer (15) combined with adoptive transfer of tumor-specific TCR transgenic $CD8^+$ T cells (TCR-I; ref. 16) as well as advanced B16 melanoma. We found that the combination of agonistic anti-CD40 + IL2/anti-IL2 complexes (IL2cx) + IL12Fc was a distinctively effective treatment with respect to priming protective, tumor-specific immunity and eradicating tumors at advanced disease stage.

Materials and Methods

Mice

C57BL/6-Tg(TRAMP)8247Ng/J (TRAMP; ref. 15) and B6.Cg-Tg(TcraY1,TcrbY1)416Tev/J (TCR-I) mice (16) were purchased from The Jackson Laboratory. B6;D2-Tg(TcrLCMV)327Sdz (P14) and C57BL/6-Tg(TcraTcrb)1100Mjb/J (OT-I) mice were obtained from the Laboratory Animal Service Center (University of Zurich, Zurich, Switzerland) and maintained on C57BL/6 background (17, 18). C57BL/6JOlaHsd mice were obtained from Harlan Laboratories. Homozygous TRAMP female mice were bred with C57BL/6JOlaHsd males, generating heterozygous TRAMP males. Age- and sex-matched non-transgenic littermates or C57BL/6JOlaHsd were used as wild-type (WT) controls. $CD45.1^+$ TCR transgenic mice were maintained heterozygous. All mice were kept under specific pathogen-free conditions at the Institute of Laboratory Animal Science (University Hospital Zurich) and received standard chow (Provimi Kliba Cat.No. 3436) and water *ad lib*. Experiments were performed in accordance with the Swiss federal and cantonal regulations on animal protection and were approved by the Swiss cantonal veterinary office (Zurich).

Tumor models

TRAMP mice carry the oncogenic SV40 large T antigen (SV40LT) as a transgene under control of the prostate-specific rat probasin promoter and show prostatic intraepithelial neoplasia at 8 weeks and adenocarcinomas at 12 weeks of age. Adenocarcinomas progress to large poorly differentiated tumors. Metastases occur in half of TRAMP mice by 24 weeks of age (19). For survival experiments, body weight measurements and pelvic palpations were performed on the mice every 1 to 2 weeks from the age of 20 weeks. Mice with palpable tumors at 20 weeks were excluded from the study. The death

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event in tumor-free survival was defined as a tumor >1 cm^3 by palpation. The death event for overall survival was defined as at least one of five of the following termination criteria: lack of flight, poor general condition, hunched back, ruffled fur, or abdominal distension. B16F10 melanoma cells (ATCC CRL-6475) and B16F10-OVA (B16F10 stably transfected to express chicken ovalbumin, kindly provided by Dr. Melody Swartz, EPFL Lausanne, Switzerland) were cultured in DMEM (GIBCO Invitrogen) supplemented with 10% FBS (Gibco), 0.1 mmol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, and antibiotics. Cells (2×10^5) were injected subcutaneously (s.c.) in 100 μL PBS, and tumors were measured with a caliper every 2 to 3 days in two dimensions. The death event was defined as tumor size reaching 150 mm^2. Mice were randomized based on tumor size on the day before the scheduled start of therapy.

Adoptive transfer of $CD8^+$ T cells

$CD8^+$ cells were isolated from spleens using CD8α MicroBeads according to the manufacturer's protocol (Miltenyi Biotec). To track cell divisions, $CD8^+$ T cells were incubated for 10 minutes at 37°C with 1 μmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich) in PBS, washed with media containing 10% FBS, and resuspended in PBS. To generate effector $CD8^+$ T cells, $CD8\alpha^+$ cells were stimulated for 3 days in RPMI-1640 supplemented with 1× MEM nonessential amino acids, antibiotics, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 10% FBS, and 0.1 mmol/L 2-mercaptoethanol (Gibco) with Dynabeads Mouse T-Activator CD3/CD28 (Life Technologies) with a bead-to-cell ratio of 1:1 and recombinant murine cytokines (60 IU/mL IL2, 1 ng/mL IL7, 10 ng/mL IL15, and 10 ng/mL IL21; Peprotech). Unless otherwise stated, 10^6 $CD8^+$ T cells were i.v. injected.

Immunotherapies

Anti-CD40 (FGK45), anti–CTLA-4 (UC10-4F10-11), and anti–PD-1 (RMP1-14) were purified from culture supernatant using protein G sepharose 4 Fast Flow (GE Healthcare) columns according to the manufacturer's protocol. Antibodies were administered at 50 μg (anti-CD40) or 250 μg (all others) in 200 μL PBS. IL2/anti-IL2 complexes were prepared with the S4B6 mAb (20), and an equivalent of 15,000 units of recombinant mouse IL2 (eBioscience) was used per injection. Recombinant mouse IL12Fc was produced (21), and bioactivity was quantified by an IFNγ secretion assay with ConA-stimulated splenocytes (22) using the following formula: specific activity (units/mg) = $10^6/ED_{50}$ (ng/mL). IL12Fc was administered in PBS at 10,000 units/kg per i.p. injection for TRAMP mice or 100 units/kg per intratumoral (i.t.) injection for subcutaneous models. All other substances were injected intraperitonially (i.p.).

Generation of recombinant vaccinia virus and infection

Vaccinia virus (VV) WR was originally obtained from Dr. B. Moss (NIH, Bethesda, MD). Recombinant VV expressing LCMV glycoprotein (rVV-G2) was originally obtained from Dr. D. Bishop (Institute of Virology, Oxford, UK). A recombinant VV expressing the SV40LT epitope I (aa 206-215) was generated (23) using NcoI/BglII-digested pSC11.3OR.2 (24) as vector and the following annealed oligonucleotides (fwd: 5′-CATGTCCGCCATCAACAATTACGCCCAGAAGCTGTA-3′, rev: 5′-GATCTACAGCTTCTGGGCGTAATTGTTGATGGCGGA-3′) as insert. Recombinant VV were generated as previously described (25). Viral titers were determined using BSC40 cells (ATCC CRL-2761). All rVV were propagated on BSC40 cells at a multiplicity of infection of 0.1. Mice received 2×10^6 plaque-forming units i.p.

Peptides

$SV40LT_{206–215}$ (SAINNYAQKL), $SV40LT_{223–231}$ (CKGVNKEYL), $SV40LT_{404–411}$ (VVYDFLKC), and LCMV $gp_{33–41}$ (KAVYNFATC; PolyPeptide Laboratories, immunograde) were dissolved in DMSO to 10 mmol/L and stored at −20°C.

Flow cytometry

Spleens and lymph nodes were mechanically homogenized. Prostates were cut into small pieces and digested for 1 hour at 37°C with agitation in RPMI containing 1 mg/mL collagenase IV and 2.6 μg/mL (6 U/mL) DNase I (Sigma-Aldrich), washed once with RPMI followed by filtration (40 μm). Cells were resuspended in PBS and stained for 20 minutes at room temperature with fluorochrome-labeled antibodies. To detect intracellular IFNγ, cells were restimulated for 5 hours at 37°C with 10 μmol/L peptide and 10 μg/mL brefeldin A (Sigma-Aldrich). In some cases, 2.5 μg/mL of FITC anti-mouse CD107a (clone 1D4B; BioLegend) was added during restimulation. Subsequently, cells were washed in PBS followed by surface staining for 20 minutes at room temperature, washed again, and fixed with 4% paraformaldehyde in PBS. Cells were washed with permeabilization buffer (20 mmol/L EDTA + 2% FBS + 0.03% NaN_3 + 0.1% saponin in PBS). Samples were incubated overnight at 4°C with APC anti-mouse IFNγ (clone XMG1.2; BioLegend). For staining of regulatory T cells (Treg), APC anti-mouse FoxP3 (clone FJK-16s; eBioscience) was used according to the manufacturer's protocol. Unless otherwise stated, the following antibodies were purchased from BioLegend: anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD8α (53-6.7), anti-CD8β (53-5.8), anti-TCR Vβ7 (TR310), anti-TCR Vα2 (B20.1; Becton Dickinson), anti-CD4 (RM4-5), anti-CD25 (PC61), and anti–PD-1 (RMP1-30 and 29F.1A12). Cells were gated on live singlets (live/dead Fixable Violet Dead Cell Stain Kit; Life technologies). Absolute counts were determined with CountBright Absolute Counting Beads (Life Technologies). Samples were measured using a CyAn ADP 9 flow cytometer (Beckman Coulter) and analyzed with FlowJo v7.6.5 software (Tree Star; Supplementary Fig. S7).

Histology

Sections (4 μm) were prepared from formalin-fixed, paraffin-embedded prostate samples. Hematoxylin and eosin staining was performed according to standard protocol. Immunohistochemistry was performed by Sophistolab AG using antibodies against CD8β (Santa Cruz Biotechnology; #sc-1144, clone M-20), cleaved caspase 3 (Cell Signaling Technology; #9661, polyclonal), and hematoxylin counterstaining. Whole slides were scanned with a Zeiss Mirax Midi slide scanner (×20 objective, NA0.8) equipped with a 3-CCD color camera (Hitachi HV-F22) and analyzed using Pannoramic viewer 1.15.4 (3DHISTECH).

Statistical analysis

Statistical analyses were performed with GraphPad Prism version 5.03 (GraphPad Software, Inc.). For comparisons, unless otherwise stated, unpaired, two-tailed Student *t* tests with systematic Welch correction were done with an alpha of 0.05. For

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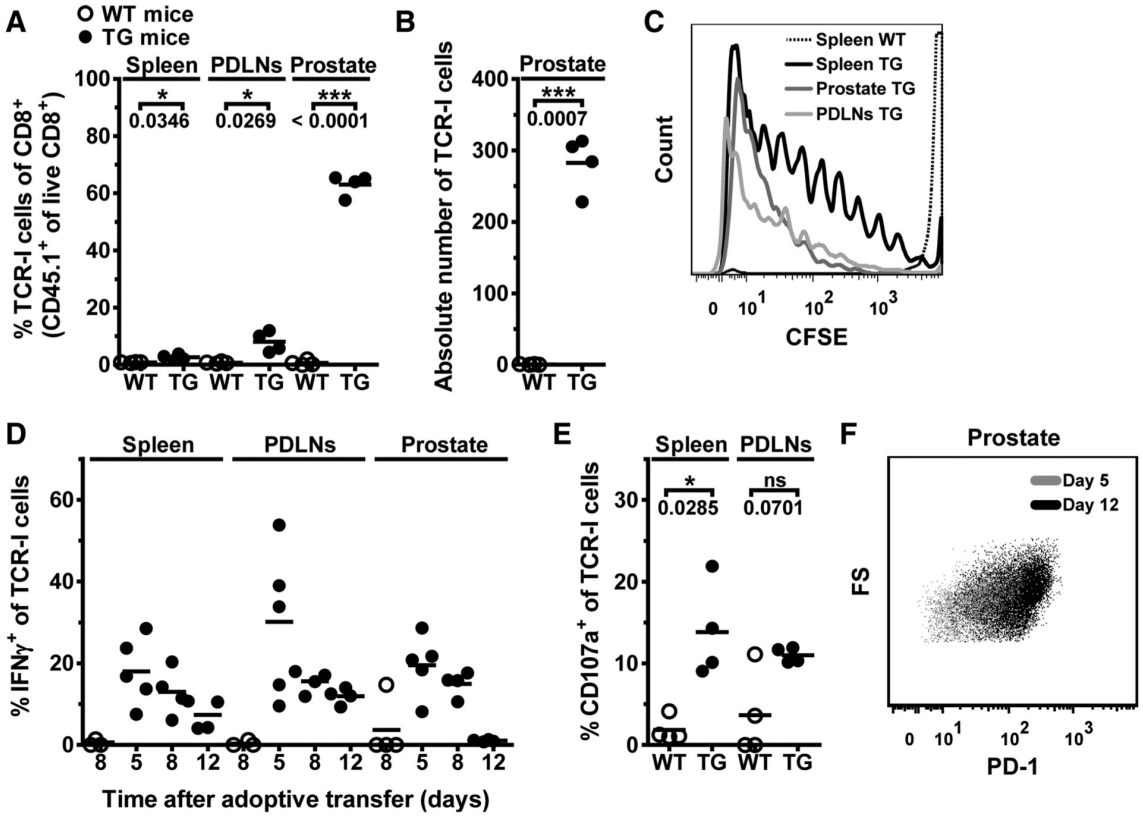

Figure 1.

TCR-I cells proliferate and migrate to the tumor, but do not develop effector function. $CD8^+$ TCR-I cells (10^6) were transferred into 13- to 20-week-old male TRAMP or WT mice. A, the percentage of TCR-I cells of $CD8^+$ T cells in spleen, PDLNs, and prostate 7 days after transfer. B, absolute number of $CD8^+$ $CD45.1^+$ TCR-I cells of live singlets per mg prostate. C, proliferation of TCR-I cells in spleen, PDLNs, and prostate 5 days after transfer. Two representative mice of 5 are shown. D, IFNγ production by TCR-I cells in spleen, PDLNs, and prostate. E, degranulation of TCR-I cells in spleen and PDLNs. F, PD-1 surface expression on TCR-I cells in the prostate. Two representative mice of 9 are shown ($n = 4$–5/group). WT, (○); TRAMP, (●). Data from one of two independent experiments are shown. Horizontal lines represent mean values *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not statistically significant. PDLN, prostate-draining lymph nodes.

comparison of more than two experimental groups, one-way ANOVA either Tukey–Kramer post-test was used to compare all pairs or Dunnett post-test to compare all groups to the control, as mentioned in figure legends. A log-rank test (Mantel–Cox) was used for Kaplan–Meier survival curves comparison between selected pairs. P values < 0.05 were considered statistically significant and marked with asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). In Fig. 3, two outliers identified with a significant Grubb test (alpha 0.05) with the online tool (http://graphpad.com/quickcalcs/Grubbs1.cfm) are shown in the bar graph, but were excluded from the statistical analysis.

Results

Tumor-specific $CD8^+$ T cells are insufficiently primed in tumor-bearing mice

To study whether tumor-specific $CD8^+$ T cells are primed to full effectors in the context of established tumors, we transferred CFSE-labeled tumor-specific $CD8^+$ T cells (TCR-I) into 13- to 14-week-old male TRAMP (TG) mice and WT mice. TCR-I cells migrated into the prostate of TRAMP mice (Fig. 1A and B) and proliferated in all organs analyzed, showing that TCR-I cells recognized their cognate antigen (Fig. 1C). However, only a small proportion of TCR-I cells produced IFNγ and degranulated ($CD107a^+$; Fig. 1D and E). Furthermore, IFNγ production decreased over time, whereas expression of PD-1 increased (Fig. 1D and F). Similar results were observed in TRAMP mice aged between 12 and 25 weeks (data not shown). Thus, in the context of cancer, $CD8^+$ T cells are inefficiently primed and progressively lose their already limited effector function. Priming of transferred TCR-I cells was restored by anti-CD40 antibodies (Supplementary Fig. S1), suggesting that insufficient DC maturation precludes the development of effector function of tumor-specific $CD8^+$ T cells in TRAMP mice.

Naïve and effector tumor-specific $CD8^+$ T cells lose function in tumor-bearing mice

To investigate whether tolerance induction is antigen dependent, we cotransferred TCR-I and P14 cells (as control) into WT and TRAMP mice followed by infection with two recombinant VVs, one encoding $SV40LT_{206\text{-}215}$ (rVV-I), the other LCMV gp (rVV-G2), to trigger responses in both transferred populations (Fig. 2A). Whereas a high proportion of TCR-I and P14 cells in the spleen and prostate-draining lymph nodes (PDLN) of WT

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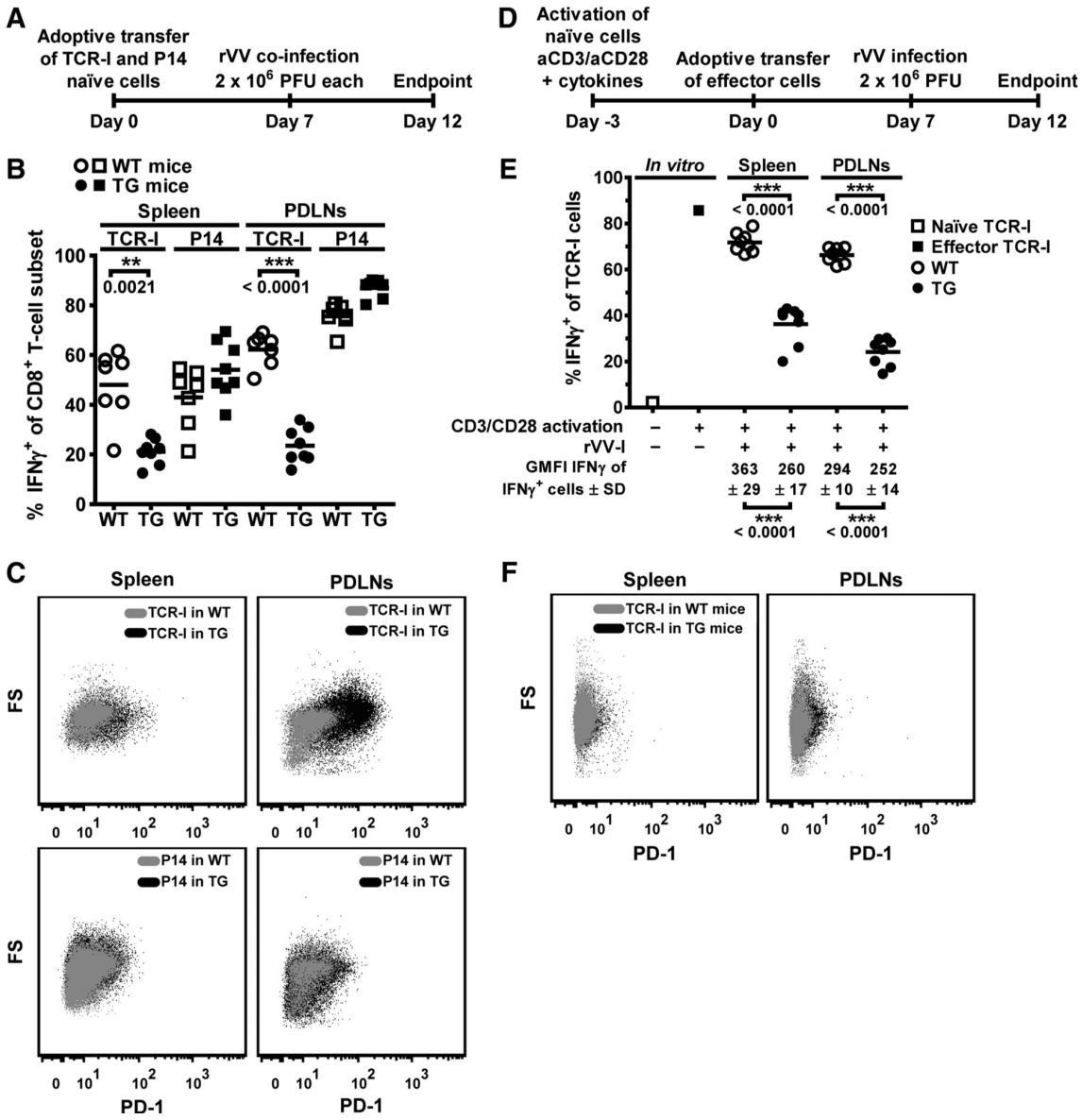

Figure 2.

Tumor-specific naïve and effector CD8$^+$ T cells are tolerized in TRAMP mice. A, 10^6 TCR-I (CD45.1$^+$2$^-$) + 10^6 P14 (CD45.1$^+$2$^+$) CD8$^+$ T cells were transferred into 14- to 15-week-old male TRAMP and WT mice. Mice were infected with 2×10^6 PFU rVV-I + 2×10^6 PFU rVV-G2. B, IFNγ production and C, representative examples for PD-1 surface expression of TCR-I and P14 cells in the spleen and PDLNs. D, CD8$^+$ TCR-I effector cells (5×106) were transferred into 12- to 14-week-old male TRAMP or WT mice and infected with 2×10^6 PFU rVV-I. E, frequency and intensity of IFNγ production by TCR-I cells in spleen and PDLNs. F, representative examples of PD-1 surface expression on TCR-I cells. Data from two of four independent experiments are shown ($n = 7$–8/group); **, $P < 0.01$; ***, $P < 0.001$. Horizontal lines represent mean values.

mice produced IFNγ, responses of TCR-I, but not P14 cells, were significantly lower in TRAMP mice (Fig. 2B and Supplementary Fig. S2A–S2L). PD-1 surface expression was elevated only on TCR-I cells in TRAMP mice (Fig. 2C). Thus, antigen encounter by CD8$^+$ T cells in the context of established cancer induces robust T-cell tolerance, which cannot be overcome by subsequent viral challenge.

Because adoptive T-cell therapy is a promising approach to treat cancer (8), we transferred tumor-specific CD8$^+$ effector T cells, challenged mice 7 days later with rVV-I, and assessed TCR-I function on day 12 (Fig. 2D). Both in spleen and PDLNs of TRAMP mice, effector TCR-I cells displayed a 2-fold reduction of the frequency of IFNγ$^+$ cells compared with WT mice and produced significantly less IFNγ on a per-cell basis (Fig. 2E), although

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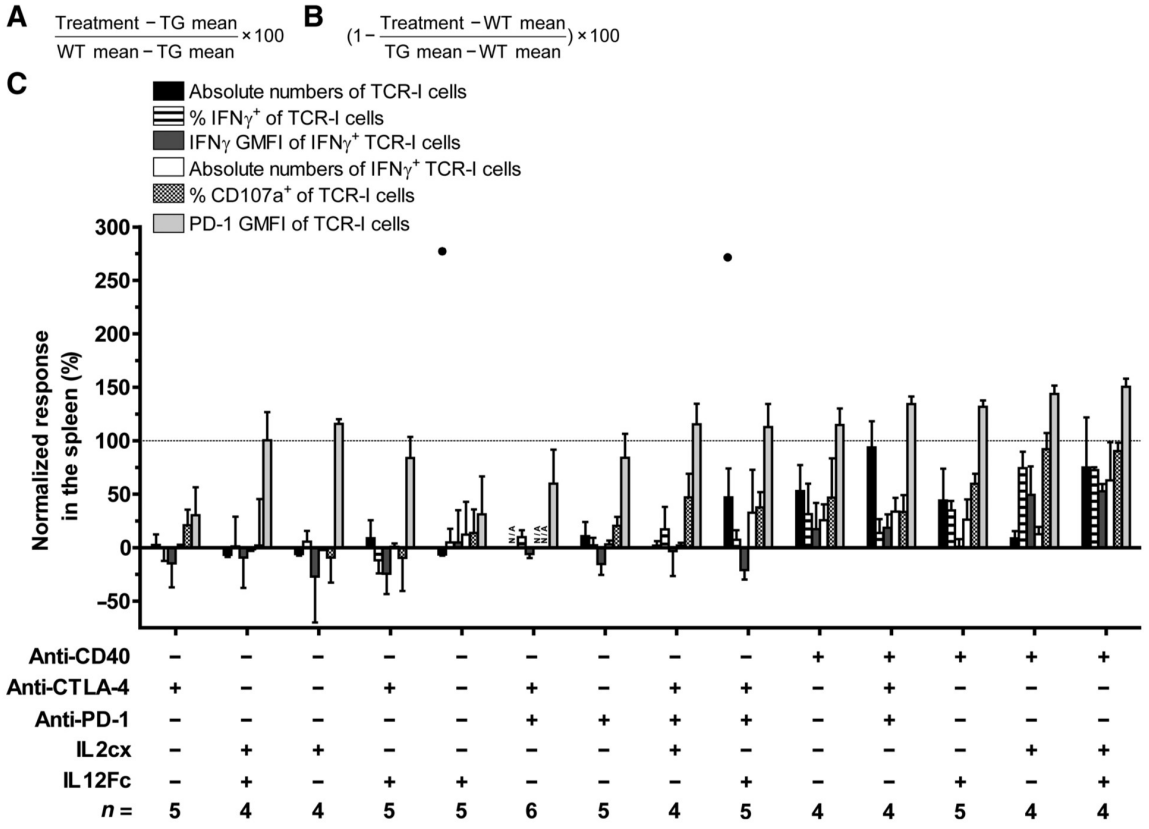

Figure 3.

Preventing tolerance induction by immune intervention. $CD8^+$ TCR-I cells (10^6) were transferred into 13- to 17-week-old male TRAMP or WT mice. Mice were treated as described in Supplementary Fig. S3A and infected 7 days after transfer with 2×10^6 PFU rVV-I. A, normalization of data. B, normalization of PD-1 expression data. C, normalized responses obtained from 14 different immunotherapies for 6 read-outs in the spleen. Data are represented as mean ± SD. Two outliers identified by a significant Grubb test were excluded from the calculations but are shown on the bar graph (●).

prior activation of TCR-I cells prevented PD-1 upregulation *in vivo* on effector (Fig. 2F) but not naïve cells (Fig. 2C). Thus, naïve and, to a lesser extent, effector tumor-specific $CD8^+$ T cells rapidly lose function in tumor-bearing mice.

Preventing tolerance induction by immune intervention

We used challenge with rVV-I to identify interventions that prevent tolerance induction (Supplementary Fig. S3A) and tested different treatments, including anti-CD40, IL2cx, IL12Fc, and blockade of CTLA-4 and PD-1. Data from three independent experiments were pooled, and for the purpose of comparison, we normalized the results to responses without intervention in WT (100%) and TRAMP mice (0%) for each of the six read-outs (Fig. 3A–C). Depending on the read-out, the individual treatments had different rankings (Fig. 3C and Supplementary Table S1), thus providing a rationale for combined use. Anti-CD40 was present in the five best treatments and was also the most potent single-agent therapy, underscoring the importance of DC activation. Anti-CD40+IL2cx+IL12Fc was the most potent regimen tested overall with respect to TCR-I numbers, IFNγ production, degranulation, and concomitant reduction in PD-1 expression (Fig. 3C; Supplementary Fig. S3B–S3E; and Supplementary Table S1). Thus, simultaneous targeting of multiple pathways prevents the induction of tumor-specific $CD8^+$ T-cell tolerance in tumor-bearing mice.

Treatment with anti-CD40+IL2cx+IL12Fc turns tolerance of tumor-specific immunity into priming

To investigate whether anti-CD40+IL2cx+IL12Fc promotes priming of tumor-specific $CD8^+$ T cells, we transferred naïve TCR-I cells into TRAMP mice and concomitantly started therapy (Fig. 4A). One week after transfer, we found a statistically significant increase in absolute numbers of TCR-I cells and in the frequency and quality of IFNγ-producing TCR-I cells in the periphery and tumor of treated mice (Fig. 4B and Supplementary Fig. S4A), and high surface PD-1 expression was prevented upon treatment (Supplementary Fig. S4B). In addition, immunohistochemistry confirmed the flow cytometry data shown in Supplementary Fig. S4A and S4B: Treatment with anti-CD40+IL2cx+IL12Fc resulted in higher infiltration of the prostate by $CD8\beta^+$ cells at day 7 after adoptive transfer of TCR-I cells (Supplementary Fig. S4G). On day 21 after transfer, hardly any $CD8\beta^+$ cells were found in the control group, whereas $CD8\beta^+$ cells infiltrated the tumor in mice treated with anti-CD40+IL2cx+IL12Fc (Supplementary Fig. S4H). Also, anti-CD40+IL2cx+IL12Fc outperformed all other combinations tested with this experimental setup (Supplementary Fig. S5A–S5F).

To investigate whether anti-CD40+IL2cx+IL12Fc can rescue tolerized TCR-I cells, we started treatment 2 weeks after

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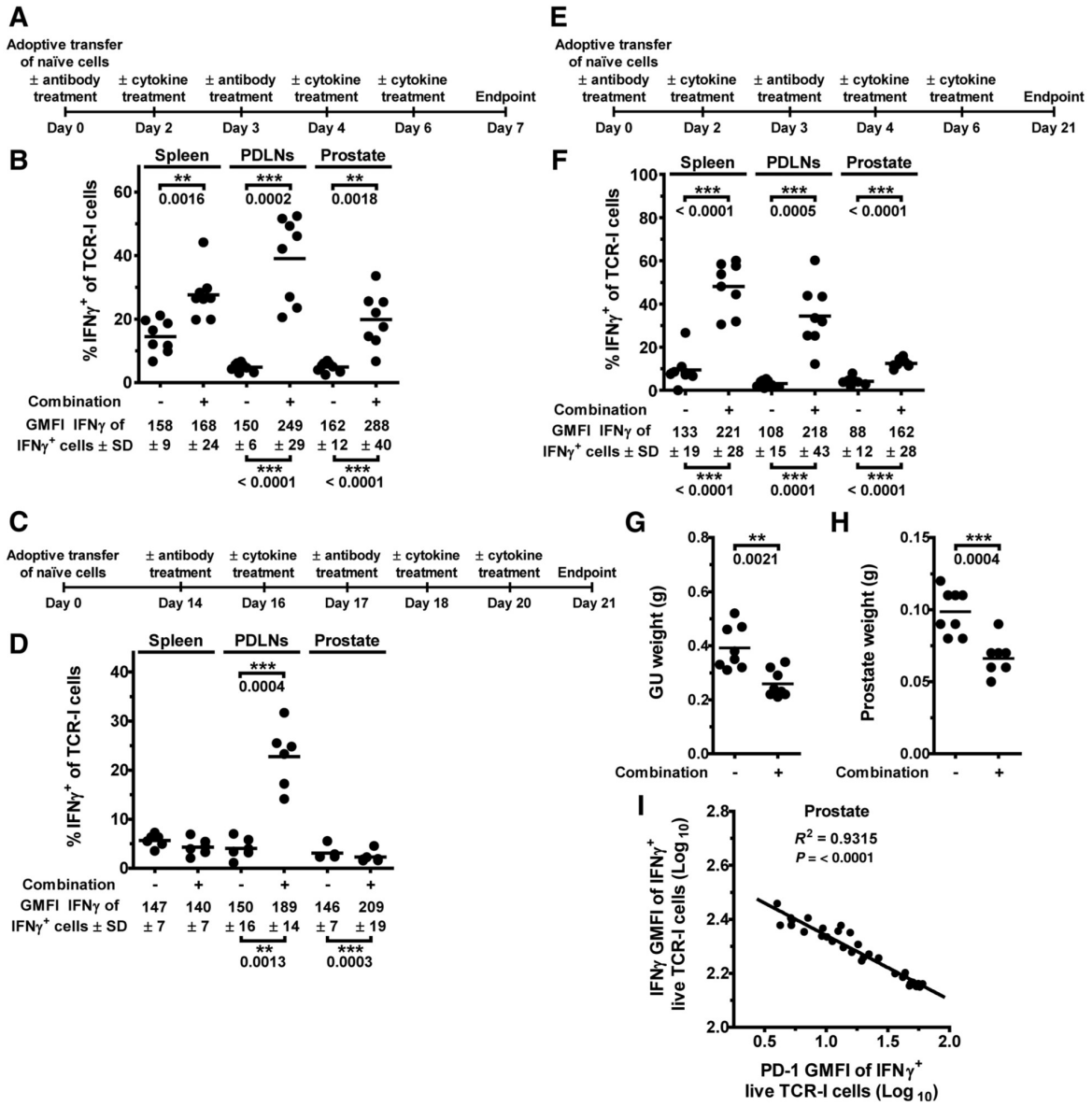

Figure 4.

Administration of anti-CD40+IL2cx+IL12Fc improves tumor-specific immunity. Naïve TCR-I cells (10^6) were transferred into 16- to 18-week-old male TRAMP mice. A and B, prevention of tolerance. B, D, and F, frequency and intensity of IFNγ production by TCR-I cells in the spleen, PDLNs, and prostate. C and D, rescue of tolerized TCR-I cells. E–H, sustained responses by TCR-I cells. G, weight of the genitourinary tract (seminal vesicles + prostate gland) on day 21. H, weight of prostate (day 21). I, logarithmic transformation of PD-1 geometric mean fluorescence intensity (GMFI) and IFNγ GMFI gated on IFNγ$^+$ TCR-I cells was plotted with prostate samples from A, C, and E. Samples with less than 20 IFNγ$^+$ TCR-I cells were excluded from the correlation. Symbols represent individual mice (n = 6–8/group). Data from three of seven independent experiments are shown; **, $P < 0.01$; ***, $P < 0.001$. Horizontal lines represent mean values.

adoptive transfer and performed analysis a week later (Fig. 4C). Anti-CD40+IL2cx+IL12Fc rescued tolerized TCR-I cells with respect to their numbers (Supplementary Fig. S4C), the frequency of IFNγ-producing TCR-I in the PDLNs, and the per-cell production of IFNγ in PDLNs and prostate (Fig. 4D). A proportion of TCR-I cells in the prostate expressed lower levels of PD-1 in treated animals (Supplementary Fig. S4D).

To investigate whether anti-CD40+IL2cx+IL12Fc induces sustained tumor-specific immunity, we treated adoptively transferred mice for a week and analyzed the data 2 weeks later (Fig. 4E). Again, the treated group showed superior responses in terms of absolute numbers (Supplementary Fig. S4E), frequency and intensity of IFNγ production (Fig. 4F), and PD-1 expression (Supplementary Fig. S4F) by TCR-I cells. On day 21 after the start

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of the therapy, the treated mice had significantly reduced tumor burden (Fig. 4G and H). This reduction was not observed 1 week after treatment (Fig. 4A) or when treatment was started 2 weeks after adoptive transfer (Fig. 4C). When combining the data from these three different setups (Fig. 4A, C, and E), we found an inverse correlation between IFNγ and PD-1 geometric mean fluorescence intensity (GMFI) restricted to IFNγ$^+$ TCR-I cells, with the strongest association ($R^2 = 0.9315$) in the prostate (Fig. 4I). Thus, treatment with anti-CD40+IL2cx+IL12Fc leads to clinical responses with durable local and systemic immunity. Moreover, tolerized tumor-specific CD8$^+$ T cells were rescued to a limited extent, albeit without impact on tumor burden at the time point investigated.

To investigate the impact of the treatment on endogenous tumor-specific CD8$^+$ T cells, we used 3 H-2^b–restricted CD8 epitopes derived from SV40LT (26). Mice were treated as described (Fig. 4A and C), and single-cell suspensions from spleen, PDLNs, and prostate were stimulated *in vitro* with the three pooled SV40LT peptides, followed by intracellular staining for IFNγ. Seven days after adoptive transfer, the frequency of IFNγ$^+$ endogenous CD8$^+$ cells was significantly higher in treated mice (Fig. 5A), as were absolute counts of endogenous CD8$^+$ T cells in the prostate (Fig. 5B). However, this effect was no longer detected 2 weeks after cessation of therapy. Higher numbers of IFNγ-producing endogenous CD8$^+$ T cells correlated with higher surface PD-1 levels as compared with the control group (Fig. 5C), in contrast with the observations with TCR-I cells (Fig. 4C and Supplementary Fig. S5). The highest endogenous CD8$^+$ to Treg ratio was observed on day 21 (Fig. 5D), but this was due in part to the low number of Tregs in the prostate (Fig. 5E). Because we only analyzed the response against three SV40LT-derived epitopes, we most likely underestimated the endogenous CD8$^+$ T-cell response to the tumor. In addition, the absolute number of endogenous CD8$^+$ T cells and the endogenous CD8/Treg ratio in the prostate increased upon treatment with anti-CD40 (Supplementary Fig. S1B and S1E). Thus, anti-CD40+IL2cx+IL12Fc enhances endogenous tumor-specific immunity.

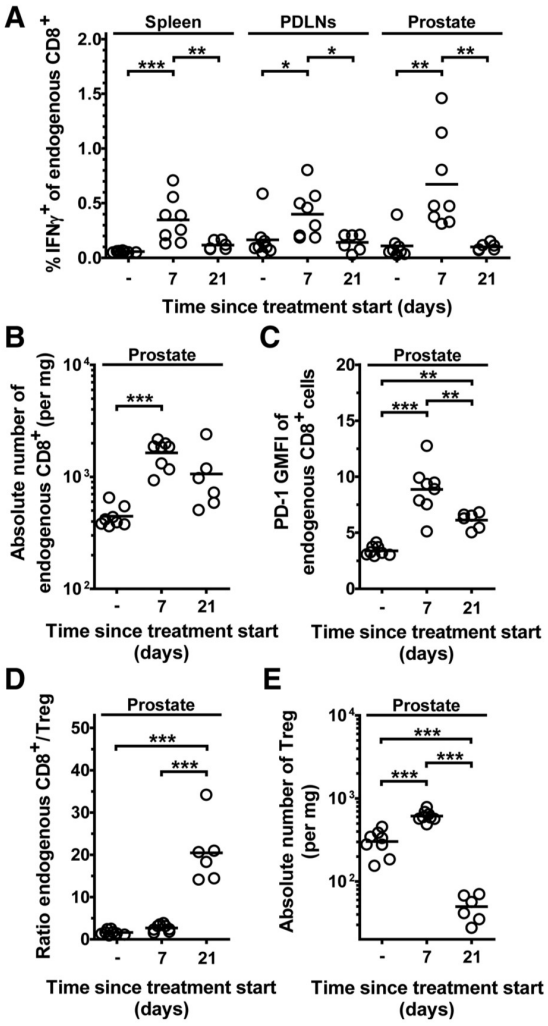

Figure 5.
Administration of anti-CD40+IL2cx+IL12Fc improves endogenous tumor-specific immunity. Endogenous CD8$^+$ (CD45.1$^-$) cells were stimulated for 5 hours with a combination of 3 SV40LT-derived peptides. A, frequency of IFNγ production by CD8$^+$ T cells. B, absolute number and C, surface PD-1 expression on CD8$^+$ T cells. D, ratio of absolute number of CD8$^+$ cells to FoxP3$^+$ CD4$^+$ Treg cells. E, absolute number of FoxP3$^+$ CD4$^+$ Treg cells in the prostate. Symbols represent individual mice (n = 7–8/group). Data from one of two independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Statistical analysis was done by one-way ANOVA and Tukey–Kramer post-test to compare all pairs per organ. Horizontal lines represent mean values.

Clinical efficacy of anti-CD40+IL2cx+IL12Fc in advanced cancer

We investigated the clinical efficacy of the therapy for advanced tumors using TRAMP mice and subcutaneously injected B16F10 syngeneic melanoma that does or does not express ovalbumin (OVA; Fig. 6A and E).

TRAMP mice were treated at 20 weeks when all mice have advanced cancer (19). Although the control group had a median tumor-free survival of 32.3 weeks and median overall survival of 37.6 weeks, mice that received either TCR-I cells alone or TCR-I cells + anti-CD40+IL2cx+IL12Fc had significantly prolonged survival (Fig. 6B and C). Moreover, 38% (5/13) of the mice receiving TCR-I cells + anti-CD40+IL2cx+IL12Fc remained tumor-free and 85% (11/13) were alive at 68 weeks of age (tumor-free and overall survival, $P < 0.0001$ vs. control). Most importantly, tumor-free survival and overall survival of mice treated with TCR-I cells + anti-CD40+IL2cx+IL12Fc were significantly improved compared with adoptive transfer alone ($P = 0.0012$ and $P < 0.0001$, respectively). The combination therapy required concomitant adoptive transfer of TCR-I cells to have clinical efficacy in this late-stage autochthonous prostate cancer. A 1-week treatment with anti-CD40+IL2cx+IL12Fc with concomitant adoptive transfer of TCR-I cells induced long-lasting protective immunity, as transferred TCR-I cells were detectable in blood of treated mice more than 6 months after adoptive transfer, but not in mice receiving only TCR-I cells. Although comparison was not possible at this late time point, peripheral TCR-I cells remained functional in treated animals in terms of IFNγ secretion and degranulation when restimulated with SV40LT$_{206–215}$ peptide (Fig. 6D). Thus, the combination therapy synergized with transferred TCR-I cells to eradicate advanced cancer in TRAMP mice and provided protective, long-lasting tumor-specific immunity.

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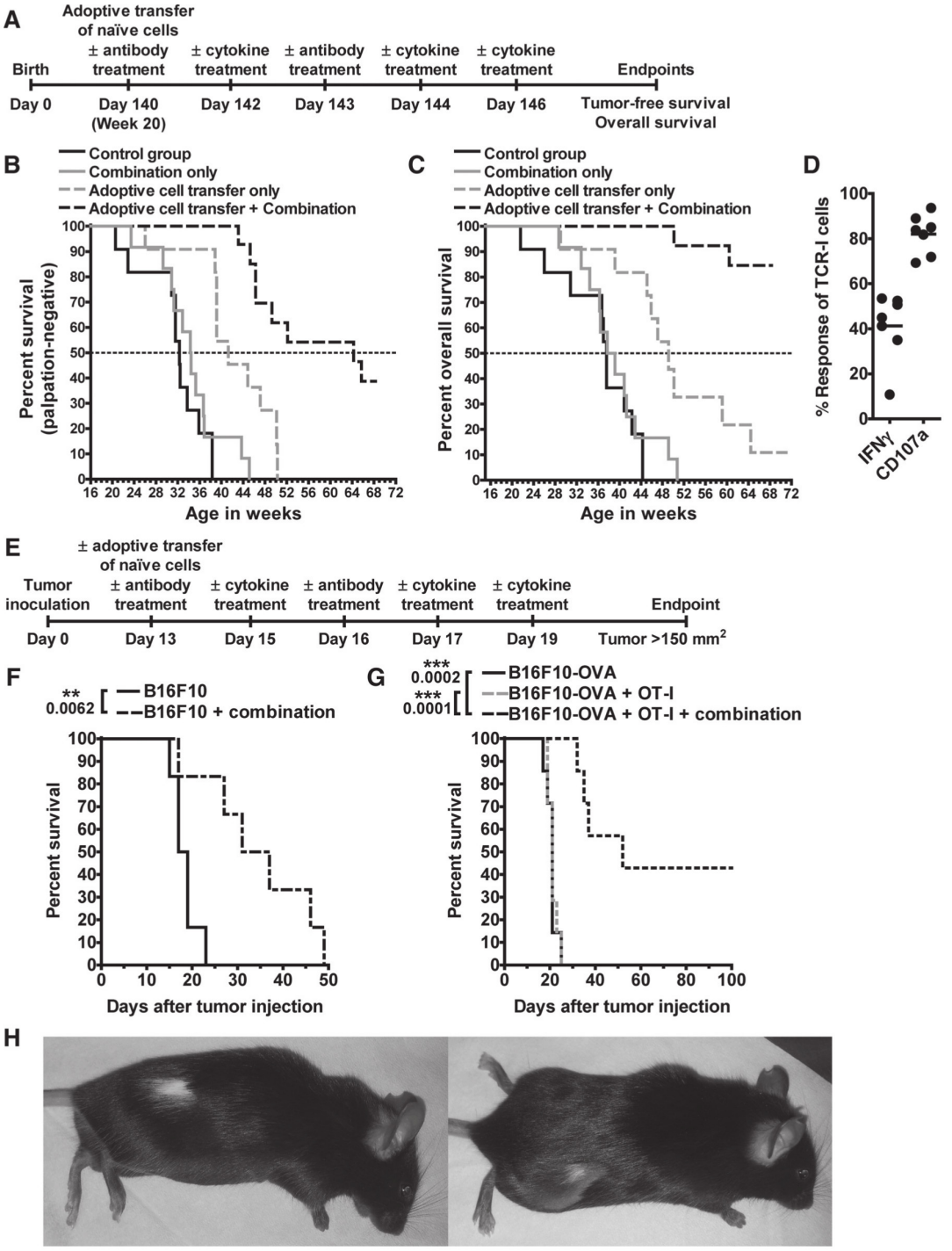

Figure 6.
Anti-CD40+IL2cx+IL12Fc controls late-stage cancer. A, treatment schedule for TRAMP mice. B, tumor-free survival of TRAMP mice. C, overall survival of TRAMP mice. D, frequency of IFNγ$^+$ and CD107$^+$ TCR-I cells 6 months after immune intervention. E, experimental design for late-stage treatment of subcutaneous B16 melanoma. F, Kaplan–Meier survival analysis of mice with B16F10 tumors. G, Kaplan–Meier survival analysis of mice bearing B16F10-OVA tumors, some of which received 10^6 naïve OT-I CD8$^+$ cells. H, two of three cured mice from G show vitiligo at the site of the rejected tumor (day 100). TRAMP survival analysis was performed once ($n = 11$–14/group). Data from two of four experiments are shown for the subcutaneous models ($n = 6$–7/group). **, $P < 0.01$; ***, $P < 0.001$. Log-rank (Mantel–Cox) tests were performed between pairs as described.

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Treatment of B16F10 or B16F10-OVA tumors started on day 13 after tumor injection (tumor size ~40–50 mm^2), a time at which single reagents cannot control tumor growth (27 and unpublished data). The median survival of B16F10-bearing mice treated with anti-CD40+IL2cx+IL12Fc nearly doubled (34 days) compared with the controls (18 days; Fig. 6F and Supplementary Fig. S6A).

Some of B16F10-OVA–bearing mice received adoptively transferred OT-I cells intravenously on day 13. Without anti-CD40+IL2cx+IL12Fc, mice with B16F10-OVA tumors had a median survival time of 21 days irrespective of OT-I transfer. Treatment with anti-CD40+IL2cx+IL12Fc + OT-I cells prolonged the median survival to 52 days and cured 43% (3/7) of the mice (Fig. 6G and Supplementary Fig. S6B). Tumor rejection was accompanied by local vitiligo, indicating an endogenous immune response against melanocytes (Fig. 6H). Thus, supporting tumor-specific $CD8^+$ T cells on three levels (anti-CD40, IL2cx and IL12Fc) generates protective immunity that can eradicate advanced tumors.

Discussion

The clinical response to immune-modulating drugs, such as anti-CTLA-4 and -PD-1, illustrates that the immune system can be exploited to manage cancer (12, 28, 29). However, only a fraction of the patients respond to such therapies, suggesting that additional pathways must be targeted to improve clinical efficacy.

We identified the combination of anti-CD40+IL2cx+IL12Fc as uniquely efficacious in inducing sustained tumor-specific immunity that controls or cures advanced cancer when combined with adoptive T-cell transfer. Without intervention, naïve TCR-I cells proliferate upon transfer into TRAMP mice, but are subsequently tolerized, suggesting that signals 2 (costimulation) and 3 (cytokines) rather than signal 1 (TCR) are limiting.

A major result of concomitant TCR and CD28 signaling is the production of IL2 by T cells. However, IL2 production is transient and Tregs form a sink for IL2 (30, 31), which may be especially relevant in the Treg-rich tumor microenvironment. Specific support to effector T cells is possible by using IL2cx, which targets IL2 to antigen-experienced $CD122^{high}$ (IL2Rβ) T cells (20).

While anti-CD40+IL2cx supports priming and expansion of tumor-specific $CD8^+$ T cells in the context of advanced cancer, their differentiation into protective effectors requires additional signals, such as IL12 (5). Accordingly, combining anti-CD40+IL2cx with IL12Fc significantly improved the quality of tumor-specific T cells with respect to many parameters. Together, our data are in line with recent work showing that T-cell division destiny depends on the integrated quality of signals 1, 2, and 3 (32).

Although APC maturation is essential and usually sufficient to prevent the induction of peripheral T-cell tolerance, the immunosuppressive tumor microenvironment poses an additional challenge. PD-1 was originally described as an activation marker on T cells (33), but later data showed that PD-1 acts as a coinhibitory molecule (34) involved in peripheral T-cell tolerance (35). TCR-I cells progressively upregulated the expression of PD-1 upon adoptive transfer into TRAMP mice, which negatively correlated with IFNγ production, in agreement with PD-1 marking T-cell exhaustion/dysfunction (36). However, endogenous $CD8^+$ T cells behaved differently with respect to PD-1 expression: Treatment with anti-CD40+IL2cx+IL12Fc resulted in increased IFNγ production and PD-1 expression. Because the expression of PD-1 on endogenous $CD8^+$ T cells in TRAMP mice treated with anti-CD40+IL2cx+IL12Fc is substantially lower than that on TCR-I cells in untreated TRAMP mice, we think that intermediate expression of PD-1 marks T-cell activation, whereas high expression indicates exhaustion/dysfunction (37). The greater tendency of TCR-I cells to express high levels of PD-1 in the context of advanced cancer when compared with endogenous $CD8^+$ T cells may be related to TCR affinity (38). All interventions tested here had an impact on PD-1 expression by TCR-I cells, pointing toward PD-1 as a central player in the negative feedback mechanism following activation. However, blocking PD-1 or reducing its surface expression seems insufficient to induce protective immunity.

Recent studies have suggested that the efficacy of checkpoint blockade depends on *de novo* priming toward tumor-specific mutant antigens (39, 40). Our study supports the importance of *de novo* priming by showing that rescuing preexisting tumor-infiltrating $CD8^+$ T cells is inefficient. Preexisting immunity is presumably compromised and cannot be sufficiently boosted, but, instead, efficacy may rely on new thymic emigrants (or transferred T cells) that encounter tumor-specific antigens in an immunogenic context. Indeed, our data show that treatment with anti-CD40+IL2cx+IL12Fc during the first week of adoptive transfer results in clinical efficacy and maintenance of protective effector TCR-I cells over a period of at least 6 months in TRAMP mice with advanced cancer. Therefore, combining anti-CD40+IL2cx+IL12Fc with adoptively transferred, tumor-specific T cells may be a promising approach to translate into clinical practice, for example, in the context of transferred autologous T cells, which express engineered TCRs or chimeric antigen receptors (CAR).

In conclusion, we have identified a novel therapeutic intervention for advanced tumors that durably supports tumor-specific immunity and shows clinical responses in multiple cancer models. The efficacy of anti-CD40+IL2cx+IL12Fc outperformed all other treatments we tested here, and we think that combined improvement of signals 2 and 3 explains this result.

We propose that an optimal cancer immunotherapy based on T cells should tackle three major obstacles. First, the frequency of fresh tumor-specific T cells needs to be increased. Second, appropriate costimulation must be provided. Third, survival and differentiation of tumor-infiltrating T cells must be supported by (local) cytokines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Bransi, B. Becher, A. Knuth, M. van den Broek
Development of methodology: A. Bransi, K. Milo, M. van den Broek
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Bransi, O.C. Salgado, M. Beffinger, H. Yagita, A. Knuth
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Bransi, O.C. Salgado, A. Knuth, M. van den Broek
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Other (review of the article): O.C. Salgado
Other (supported in generating data): A. Bransi
Other (experiments for paper revision and Figure S4G): K. Silina

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7. Curriculum vitae

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